

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 May 2003 (22.05.2003)

PCT

(10) International Publication Number
WO 03/042244 A2

- (51) International Patent Classification⁷: C07K 14/54 (74) Agent: KOEFOED, Peter; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK).
- (21) International Application Number: PCT/DK02/00764
- (22) International Filing Date:
15 November 2002 (15.11.2002)
- (25) Filing Language: English
- (26) Publication Language: English
- (30) Priority Data:
PA 2001 01702 16 November 2001 (16.11.2001) DK
60/331,575 16 November 2001 (16.11.2001) US
- (71) Applicant (for all designated States except US):
PHARMEXA A/S [DK/DK]; Kogle Allé 6, DK-2970 Hørsholm (DK).
- (71) Applicants and
(72) Inventors: KLYSNER, Steen [DK/DK]; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). NIELSEN, Finn, Stausholm [DK/DK]; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). BRATT, Tomas [DK/DK]; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). VOLDborg, Bjørn [DK/DK]; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK). MOURITSEN, Søren [DK/DK]; c/o Pharmexa A/S, Kogle Allé 6, DK-2970 Hørsholm (DK).
- (81) Designated States (national): AE, AG, AL, AM, AT (utility model), AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ (utility model), CZ, DE (utility model), DE, DK (utility model), DK, DM, DZ, EC, EE (utility model), EE, ES, FI (utility model), FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH, PL, PT, RO, RU, SC, SD, SE, SG, SI, SK (utility model), SK, SL, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.
- (84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:
— without international search report and to be republished upon receipt of that report
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: NOVEL IMMUNOGENIC MIMETICS OF MULTIMER PROTEINS

(57) Abstract: The present invention relates to novel immunogenic variants of multimeric proteins such as immunogenic variants of interleukin 5 (IL5) and tumour necrosis factor alpha (TNF, TNF α). The variants are, besides from being immunogenic in the autologous host, also highly similar to the native 3D structure of the proteins from which they are derived. Certain variants are monomeric mimics of the multimers, where peptide linkers (inert or T helper epitope containing) ensure a spatial organisation of the monomer units that facilitate correct folding. A subset of variants are monomer TNF α variants that exhibit a superior capability of assembling into multimers with a high structural similarity to the native protein. Also disclosed are methods of treatment and production of the variants as well as DNA fragments, vectors, and host cells.

WO 03/042244 A2

NOVEL IMMUNOGENIC MIMETICS OF MULTIMER PROTEINS

FIELD OF THE INVENTION

The present invention relates to the field of therapeutic immunotherapy, and in particular to the field of active immunotherapy targeted at down-regulating autologous ("self") proteins and other weakly immunogenic antigens. The invention thus provides novel and improved immunogenic variants of multimeric proteins as well as the necessary tools for the preparation of such variants. The invention further relates to methods of immunotherapy as well as compositions useful in such methods.

BACKGROUND OF THE INVENTION

Use of active immunotherapy ("vaccination") as a means of curing or alleviating disease has received growing attention over the last 2 decades. Notably, the use of active immunotherapy as a means for breaking tolerance to autologous proteins that are somehow related to a pathological (or otherwise undesired) physiologic condition has been known since the late seventies where the first experiments with antifertility vaccines were reported.

Vaccines against autologous antigens have traditionally been prepared by "immunogenizing" the relevant self-protein, e.g. by chemical coupling ("conjugation") to a large foreign and immunogenic carrier protein (cf. US 4,161,519) or by preparation of fusion constructs between the autologous protein and the foreign carrier protein (cf. WO 86/07383). In such constructs, the carrier part of the immunogenic molecule is responsible for the provision epitopes for T-helper lymphocytes

("T_H epitopes") that render possible the breaking of autotolerance.

Later research has proven that although such strategies may indeed provide for the breaking of tolerance against autologous proteins, a number of problems are encountered. Most important is the fact that the immune response that is induced over time will be dominated by the antibodies directed against the carrier portion of the immunogen whereas the reactivity against the autologous protein often declines, an effect that is particularly pronounced when the carrier has previously served as an immunogen - this phenomenon is known as carrier suppression (cf. e.g. Kaliyaperumal et al. 1995., Eur. J. Immunol 25, 3375-3380). However, when using therapeutic vaccination it is usually necessary to re-immunize several times per year and to maintain this treatment for a number of years and this also results in a situation where the immune response against the carrier portion will be increasingly dominant on the expense of the immune response against the autologous molecule.

Further problems involved when using hapten-carrier technology for breaking autotolerance is the negative steric effects exerted by carrier on the autologous protein part in such constructs: The number of accessible B-cell epitopes that resemble the conformational patterns seen in the native autologous protein is often reduced due to simple shielding or masking of epitopes or due to conformational changes induced in the self-part of the immunogen. Finally, it is very often difficult to characterize a hapten-carrier molecule in sufficient detail.

WO 95/05849 provided for a refinement of the above-mentioned hapten-carrier strategies. It was demonstrated that self-pro-

teins wherein is in-substituted as little as one single foreign T_H epitope are capable of breaking tolerance towards the autologous protein. Focus was put on the preservation of tertiary structure of the autologous protein in order to ensure
5 that a maximum number of autologous B-cell epitopes would be preserved in the immunogen in spite of the introduction of the foreign T_H element. This strategy has generally proven extremely successful inasmuch as the antibodies induced are broad-spectred as well as of high affinity and that the immune
10 response has an earlier onset and a higher titer than that seen when immunizing with a traditional carrier construct.

WO 00/20027 provided for an expansion of the above principle. It was found that introduction of single T_H epitopes in the coding sequence for self-proteins could induce cytotoxic T-
15 lymphocytes (CTLs) that reacts specifically with cells expressing the self-protein. The technology of WO 00/20027 also provided for combined therapy, where both antibodies and CTLs are induced - in these embodiments, the immunogens would still be required to preserve a substantial fraction of B-cell epi-
20 topes.

WO 95/05849 and WO 98/46642 both disclose vaccine technology that is suitable for down-regulating the activity of $TNF\alpha$ (tumour necrosis factor α), a cytokine involved in the pathology of several diseases such as type I diabetes, rheumatoid ar-
25 thritis, and inflammatory bowel disease. Both disclosures teach preservation of the tertiary structure of monomer $TNF\alpha$ when this molecule confronts the immune system.

WO 00/65058 relates to down-regulation of interleukin 5 (IL5), a molecule involved in the activation of eosinophil granulo-
30 cyte activity that is important in the pathogenesis of a num-

ber of airway diseases such as chronic asthma. It is taught that down-regulation can be accomplished by means of both polypeptide vaccination technology, live vaccines and nucleic acid vaccination and it is further taught that the preservation of B-cell epitopes is important if raising an immune response against IL5.

Even though the above-referenced technologies have provided for very promising results, there are several factors that may come into play when assessing the viability of a vaccine approach in combating a disease. One of these factors is the expression level of the immunogenic protein.

For instance, in order for a nucleic acid vaccine to be functional, the cells transfected *in vivo* with a construct encoding an "immunogenized" autologous protein must be able to express the immunogen in sufficient amounts so as to induce a suitable immune response. Also, polypeptide based vaccines require that the immunogenic protein can be produced in satisfactory amounts in an industrial fermentation process. However, it is often observed that even slight changes in the amino acid sequence of a known protein can have dramatic effects on the amounts of protein that can be recovered.

Further, the stability of genetically modified protein sequences may also be less than optimal (both in terms of shelf-life and in terms of stability *in vivo*).

Finally, when the self-protein that it is desired to down-regulate is a heteropolymer or homopolymer it is not necessarily so that a variant of a monomeric unit of this protein will be capable of inducing antibodies that are sufficiently specific for the conformation native to the polymeric protein.

OBJECT OF THE INVENTION

It is an object of the invention to provide for improved immunogenic analogues of polymeric autologous proteins as well as to provide for improved methods for inducing humoral immunity against such polymeric autologous proteins. It is a further object to provide for immunogenic analogues of self-proteins that have an improved stability and exhibit improved characteristics when expressed in heterologous host cells. Finally, it is also objects of the invention to provide for means and measures that are useful when preparing or utilising the improved immunogens.

SUMMARY OF THE INVENTION

When producing large-scale amounts of recombinant protein in bacterial host cells, it is often desired that the expression product becomes available as inclusion bodies inside the bacteria. The reasons for this are several: For example the expression yields are normally considerably higher when the protein is expressed as insoluble inclusion bodies, and the purification of the protein is also facilitated because the desired expression product is easily and conveniently separated from soluble protein from the bacterial fermentation.

When expressing a recombinant protein as insoluble inclusion bodies, it is often necessary to subject the expression product to various protein refolding processes in order to obtain it in a biologically active form, but this is normally acceptable even though such a step leads to a certain loss of total recombinant protein that is never folded into the correct biologically active form.

However, when producing recombinant immunogenic variants of non-immunogenic self-proteins it is necessary to introduce T_H epitopes and thereby the primary structure of the protein product becomes altered when compared to the native self-protein. The present inventors have experienced that even the slightest of changes renders the traditional approach of inclusion body expression followed by refolding impractical: The yields of protein after refolding that has preserved a satisfactory fraction of B-cell epitopes compared to the native self-protein are very often low, and this problem increases with the complexity of the protein in question.

It has now been found that designing and effecting expression of protein constructs that are produced as soluble protein from bacteria is a superior way of preparing immunogenic variants of self-proteins - even though subsequent purification steps become more complicated because other soluble proteins have to be removed, the final purified and correctly folded product is obtained in significantly higher yields than when compared to the traditional approach outlined above. And, very importantly, the purified proteins obtained from this type of expression exhibit a hitherto unprecedented ability to preserve B-cell epitopes of the native self-protein from which they are derived.

In brief, according to the present invention, soluble expression of variant proteins is an excellent selection criterion when initially selecting for immunogenic variants of a self-protein that are suitable for vaccination purposes.

In order to obtain the goal of soluble protein expression of such immunogenized self-proteins (and other proteins where changes have been introduced in the primary sequence), a num-

ber of parameters can be varied - multimeric proteins that are difficult to assemble can be produce by stabilising their structure both on the monomeric level but also by preparing monomeric mimicks of the multimer, and also simple monomeric
5 proteins can be stabilised according to the teachings set forth herein.

Another important factor is the fermentation conditions - findings in the present inventors' lab have e.g. indicated that fermentation of bacteria at lower temperatures than those
10 normally used for obtaining high level expression greatly facilitate the production of soluble forms of the variant proteins.

The present inventors have found that preparation of "monomerized" forms of IL5 and TNF α may provide for immunogenic molecules having a high stability, superior immunogenicity and desirable production characteristics. In particular, the yield of protein is surprisingly high when expressing recombinant polypeptides constituted by two monomers of hIL5 joined by means of a peptide linker and including foreign T helper cell
20 epitopes. It is believed that this finding constitutes a general applicable finding relating to multimeric proteins, the quarternary structure of which allows for tailoring of a monomeric version thereof.

It is believed that the present technology is especially
25 suited for preparing immunogens for breaking autotolerance against autologous proteins, since the introduction of the peptide linker can be elegantly combined with the provision of foreign T helper epitopes while at the same time preserving the 3D structure of the multimeric protein (i.e. preservation
30 of both elements from tertiary and from quarternary structure

of such a protein, by imposing the original quarternary structure on the new tertiary structure in the monomeric protein).

Hence, in one broad aspect, the invention relates to an immunogenic analogue of a polymeric protein, said polymeric protein consisting (in nature) of at least 2 monomeric units that are not joined by means of a peptide bond, wherein said analogue

- 10 a) includes substantial fragments of at least 2 monomeric units of said polymeric protein, wherein said substantial fragments are joined via peptide bonds through a peptide linker,
- b) includes at least one MHC Class II binding amino acid sequence that is heterologous to the polymeric protein, and
- 15 c) can be produced as one single expression product from a cell harbouring an expression vector encoding the analogue.

The present inventors have also found that a number of particular manipulations in the amino acid sequence of monomeric

20 TNF α results in the provision of monomer molecules that are both immunogenic and capable of attaining a functional quarternary structure, meaning that these molecules has so high degree of preserved tertiary structure that they spontaneously can form functional, receptor binding, dimers

25 and trimers, and also that these monomers are produced as soluble proteins in bacteria.

Some of these manipulations that have been performed in the TNF α protein are believed to be generally applicable for pro-

teins where it is desired to prepare a stabilised tertiary structure compared to a native protein.

A particular aspect of the invention relates to a number of variations in the TNF α monomer structure that are sufficiently non-destructive so as to allow correct folding of the TNF α monomers while at the same time introducing at least one MHC Class II binding amino acid sequence. It has e.g. been found that insertion of a foreign T_H epitope can be made in one particular loop structure in native TNF α without this having a negative impact on the expression characteristics of the protein or on the monomer's capability of forming a functional TNF α dimer or trimer. Hence, a important part of the invention relates to an immunogenic analogue of human TNF α , wherein the analogue includes at least one foreign MHC Class II binding amino acid sequence and further has the characteristic of being

- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein has been inserted or substituted at least one foreign MHC Class II binding amino acid sequence into flexible loop 3, and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein has been introduced at least one disulfide bridge that stabilises the TNF α monomer 3D structure, and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein any one of amino acids 1, 2, 3, 4, 5, 6, 7, 8, and 9 in the amino terminus have been deleted, and/or

- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein an inserted or in-substituted at least one foreign MHC Class II binding amino acid sequence into loop 1 in an intron position, and/or
- 5 - a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one foreign MHC Class II binding amino acid sequence is introduced as part of an artificial stalk region in the N-terminus of human TNF α , and/or
- 10 - a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one foreign MHC Class II binding amino acid sequence is introduced so as to stabilize the monomer structure by increasing the hydrophobicity of the trimeric interaction interface,
- 15 and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one foreign MHC Class II binding amino acid sequence flanked by glycine residues is inserted or in-substituted in the TNF α amino
- 20 acid sequence, and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one foreign MHC Class II binding amino acid sequence is inserted or in-substituted in the D-E loop, and/or
- 25 - a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one foreign MHC Class II binding amino acid sequence is inserted or in-

substituted between two identical subsequences of human TNF α , and/or

- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein at least one salt bridge
5 in human TNF α has been strengthened or substituted with a disulphide bridge, and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein solubility and/or stability towards proteolysis is enhanced by introducing mutations that mimic murine TNF α crystalline structure,
10 and/or
- a human TNF α monomer or a monomerized analogue of TNF α of the present invention, wherein potential toxicity is reduced or abolished by introduction of at least one point
15 mutation.

In general, it has been found that all of the best suited immunogenic analogues of the invention are those that are soluble proteins already at the stage when they are produced and isolated in soluble form from their recombinant host cells.

- 20 The invention further provides for nucleic acid fragments (such as DNA fragments) encoding such immunogenic analogues and also to vectors including such DNA fragments.

The invention also provides for transformed cells useful for preparing the analogues.

- 25 The invention further provides for immunogenic compositions comprising the analogous or the vectors of the invention.

Also provided by the invention are methods of treatment, where multimeric proteins are down-regulated and to treatment of specific diseases related to the particular multimeric proteins.

5 LEGEND TO THE FIGURE

Fig. 1: The p2ZOP2f insect cell expression vector.

The sequence of the vector is set forth in SEQ ID NO: 60. The vector contains a multi-cloning site (MCS) downstream the OpIE2 promoter and upstream of an OpIE2 poly A tail (OpIE2pA).
10 The marker zeocin resistance gene (ZeoR) is under the control of a second OpIE2 promoter.

DETAILED DISCLOSURE OF THE INVENTION

Definitions

In the following, a number of terms used in the present specification and claims will be defined and explained in detail in
15 order to clarify the metes and bounds of the invention.

The terms "T-lymphocyte" and "T-cell" will be used interchangeably for lymphocytes of thymic origin that are responsible for various cell mediated immune responses as well as for
20 helper activity in the humeral immune response. Likewise, the terms "B-lymphocyte" and "B-cell" will be used interchangeably for antibody-producing lymphocytes.

A "polymeric protein" is herein defined as a protein that includes at least two polypeptide chains that are not joined
25 end-to-end via a peptide bond (the term "multimeric protein" is used interchangeably therewith). Hence, polymeric proteins

may be polymers consisting of several polypeptides that are kept together in polymeric form by means of disulfide bonds and/or non-covalent binding. Also included within the term are processed pre-proteins and pro-proteins that after processing
5 include at least two free C-termini and at least two free N-termini. Finally, included within the term is also temporarily existing complexes between at least two polypeptides that may form up an unstable but yet biologically active molecular entity that has a distinct 3-dimensional structure.

10 "An immunogenic analogue" (or an "immunogenized" analogue or variant) is herein meant to designate a single polypeptide that includes substantial parts of the sequence information found in a complete polymeric protein. That is, the analogue protein of the invention includes one polypeptide chain
15 whereas a polymeric protein includes at least 2 polypeptide chains. It should be noted that the analogue may be a variation of the polymers monomeric subunit structure, but in that case, the immunogenic analogue is capable of forming polymeric protein complexes that resemble the native polymer.

20 A "monomerized" analogue or variant of a polymeric protein is in the present context a single polypeptide that includes, in covalently linked form via a peptide bond, at least 2 polypeptide chains found in a polymeric protein in nature, where these 2 polypeptide chains are not linked via a peptide bond.

25 "A substantial fragment" of a monomeric unit of a multimeric protein is intended to mean a part of a monomeric polypeptide that constitutes at least enough of the monomeric polypeptide so as to form a domain that folds up in substantially the same 3D conformation as can be found in the multimeric protein.

An "IL5 polypeptide" is herein intended to denote polypeptides having the amino acid sequence of IL5 proteins derived from humans and other mammals. Also unglycosylated forms of IL5 which are prepared in prokaryotic system are included within the boundaries of the term as are forms having varying glycosylation patterns due to the use of e.g. yeasts or other non-mammalian eukaryotic expression systems. It should, however, be noted that when using the term "an IL5 polypeptide" it is intended that the polypeptide in question is normally non-immunogenic when presented to the animal to be treated. In other words, the IL5 polypeptide is a self-protein or is a xeno-analogue of such a self-protein which will not normally give rise to an immune response against IL5 of the animal in question.

A "TNF α polypeptide" is herein intended to denote polypeptides having the amino acid sequence of TNF α proteins derived from humans and other mammals. Also unglycosylated forms of TNF α which are prepared in prokaryotic system are included within the boundaries of the term as are forms having varying glycosylation patterns due to the use of e.g. yeasts or other non-mammalian eukaryotic expression systems. It should, however, be noted that when using the term "a TNF α polypeptide" it is intended that the polypeptide in question is normally non-immunogenic when presented to the animal to be treated. In other words, the TNF α polypeptide is a self-protein or is a xeno-analogue of such a self-protein which will not normally give rise to an immune response against TNF α of the animal in question.

An "IL5 analogue" is an IL5 polypeptide which has been either subjected to changes in its primary structure and/or that is associated with elements from other molecular species. Such a

change can e.g. be in the form of fusion of an IL5 polypeptide to a suitable fusion partner (i.e. a change in primary structure exclusively involving C- and/or N-terminal additions of amino acid residues) and/or it can be in the form of insertions and/or deletions and/or substitutions in the IL5 polypeptide's amino acid sequence. Also encompassed by the term are derivatized IL5 molecules, cf. the discussion below of modifications of IL5.

A "TNF α analogue" is a TNF α polypeptide which has been either subjected to changes in its primary structure and/or that is associated with elements from other molecular species. Such a change can e.g. be in the form of fusion of a TNF α polypeptide to a suitable fusion partner (i.e. a change in primary structure exclusively involving C- and/or N-terminal additions of amino acid residues) and/or it can be in the form of insertions and/or deletions and/or substitutions in the TNF α polypeptide's amino acid sequence. Also encompassed by the term are derivatized TNF α molecules, cf. the discussion below of modifications of TNF α .

It will be understood, that IL5 and TNF α analogues also include monomeric variants that contains substantial parts of complete IL5 and TNF α multimeric proteins.

When using the abbreviations "IL5" and "TNF α " herein, this is intended as references to the amino acid sequences of mature, wildtype IL5 and TNF α (also denoted "IL5m" and "IL5wt" as well as "TNF α m" and "TNF α wt" herein), respectively. Mature human IL5 is denoted hIL5, hIL5m or hIL5wt, and murine mature IL5 is denoted mIL5, mIL5m, or mIL5wt and a similar syntax is used for TNF α . In cases where a DNA construct includes information

encoding a leader sequence or other material, this will normally be clear from the context.

The term "polypeptide" is in the present context intended to mean both short peptides of from 2 to 10 amino acid residues, 5 oligopeptides of from 11 to 100 amino acid residues, and polypeptides of more than 100 amino acid residues. Furthermore, the term is also intended to include proteins, i.e. functional biomolecules comprising at least one polypeptide; when comprising at least two polypeptides, these may form complexes, 10 be covalently linked, or may be non-covalently linked. The polypeptide(s) in a protein can be glycosylated and/or lipidated and/or comprise prosthetic groups.

The term "subsequence" means any consecutive stretch of at least 3 amino acids or, when relevant, of at least 3 nucleotides, 15 tides, derived directly from a naturally occurring IL5 amino acid sequence or nucleic acid sequence, respectively.

The term "animal" is in the present context in general intended to denote an animal species (preferably mammalian), such as *Homo sapiens*, *Canis domesticus*, etc. and not just one 20 single animal. However, the term also denotes a population of such an animal species, since it is important that the individuals immunized according to the method of the invention all harbour substantially the same IL5 allowing for immunization of the animals with the same immunogen(s). If, for instance, 25 genetic variants of IL5 or TNF α exist in different human populations it may be necessary to use different immunogens in these different populations in order to be able to break the autotolerance towards IL5 and TNF α , respectively, in each population. It will be clear to the skilled person that an 30 animal in the present context is a living being which has an

immune system. It is preferred that the animal is a vertebrate, such as a mammal.

By the term "down-regulation" is herein meant reduction in the living organism of the biological activity of the multimeric protein (e.g. by interference with the interaction between the multimeric protein and biologically important binding partners for this molecule). The down-regulation can be obtained by means of several mechanisms: Of these, simple interference with the active site in the multimeric protein by antibody binding is the most simple. However, it is also within the scope of the present invention that the antibody binding results in removal of the multimeric protein by scavenger cells (such as macrophages and other phagocytic cells).

The expression "effecting presentation ... to the immune system" is intended to denote that the animal's immune system is subjected to an immunogenic challenge in a controlled manner. As will appear from the disclosure below, such challenge of the immune system can be effected in a number of ways of which the most important are vaccination with polypeptide containing "pharmaccines" (i.e. a vaccine which is administered to treat or ameliorate ongoing disease) or nucleic acid "pharmaccine" vaccination. The important result to achieve is that immune competent cells in the animal are confronted with the antigen in an immunologically effective manner, whereas the precise mode of achieving this result is of less importance to the inventive idea underlying the present invention.

The term "immunogenically effective amount" has its usual meaning in the art, i.e. an amount of an immunogen which is capable of inducing an immune response which significantly en-

gages pathogenic agents which share immunological features with the immunogen.

When using the expression that the IL5, TNF α or other self-protein has been "modified" is herein meant a chemical modification of the polypeptide which constitutes the backbone of the self-protein. Such a modification can e.g. be derivatization (e.g. alkylation, acylation, esterification etc.) of certain amino acid residues in the amino acid sequence, but as will be appreciated from the disclosure below, the preferred modifications comprise changes of (or additions to) the primary structure of the amino acid sequence.

When discussing "autotolerance towards an autologous protein" it is understood that since the relevant multimeric protein is a self-protein in the population to be vaccinated, normal individuals in the population do not mount an immune response against it; it cannot be excluded, though, that occasional individuals in an animal population might be able to produce antibodies against the native multimer, e.g. as part of an autoimmune disorder. At any rate, an animal species will normally only be autotolerant towards its own multimer, but it cannot be excluded that analogues derived from other animal species or from a population having a different phenotype would also be tolerated by said animal.

A "foreign T-cell epitope" (or: "foreign T-lymphocyte epitope") is a peptide which is able to bind to an MHC molecule and which stimulates T-cells in an animal species - an alternate term is therefore. Preferred foreign T-cell epitopes in the invention are "promiscuous" (or "universal" or "broad-range") epitopes, i.e. epitopes that bind to a substantial fraction of a particular class of MHC molecules in an animal

species or population. Only a very limited number of such promiscuous T-cell epitopes are known, and they will be discussed in detail below. It should be noted that in order for the immunogens which are used according to the present invention to be effective in as large a fraction of an animal population as possible, it may be necessary to 1) insert several foreign T-cell epitopes in the same analogue or 2) prepare several analogues wherein each analogue has a different promiscuous epitope inserted. It should be noted also that the concept of foreign T-cell epitopes also encompasses use of cryptic T-cell epitopes, i.e. epitopes which are derived from a self-protein and which only exerts immunogenic behaviour when existing in isolated form without being part of the self-protein in question.

15 A "foreign T helper lymphocyte epitope" (a foreign T_H epitope) is a foreign T cell epitope which binds an MHC Class II molecule and can be presented on the surface of an antigen presenting cell (APC) bound to the MHC Class II molecule.

An "MHC Class II binding amino acid sequence that is heterologous to a multimeric protein" is therefore an MHC Class II binding peptide that does not exist in the multimeric protein in question. Such a peptide will, if it is also truly foreign to the animal species harbouring the multimeric protein, be a foreign T_H epitope.

25 A "functional part" of a (bio)molecule is in the present context intended to mean the part of the molecule which is responsible for at least one of the biochemical or physiological effects exerted by the molecule. It is well-known in the art that many enzymes and other effector molecules have an active site which is responsible for the effects exerted by the mole-

30

cule in question. Other parts of the molecule may serve a stabilizing or solubility enhancing purpose and can therefore be left out if these purposes are not of relevance in the context of a certain embodiment of the present invention. However, according to the present invention, it is preferred to utilise as much of the polymeric molecule as possible, because the increased stability has in fact been demonstrated when using the monomers described herein.

The term "adjuvant" has its usual meaning in the art of vaccine technology, i.e. a substance or a composition of matter which is 1) not in itself capable of mounting a specific immune response against the immunogen of the vaccine, but which is 2) nevertheless capable of enhancing the immune response against the immunogen. Or, in other words, vaccination with the adjuvant alone does not provide an immune response against the immunogen, vaccination with the immunogen may or may not give rise to an immune response against the immunogen, but the combination of vaccination with immunogen and adjuvant induces an immune response against the immunogen which is stronger than that induced by the immunogen alone.

"Targeting" of a molecule is in the present context intended to denote the situation where a molecule upon introduction in the animal will appear preferentially in certain tissue(s) or will be preferentially associated with certain cells or cell types. The effect can be accomplished in a number of ways including formulation of the molecule in composition facilitating targeting or by introduction in the molecule of groups which facilitates targeting. These issues will be discussed in detail below.

"Stimulation of the immune system" means that a substance or composition of matter exhibits a general, non-specific immunostimulatory effect. A number of adjuvants and putative adjuvants (such as certain cytokines) share the ability to stimulate the immune system. The result of using an immunostimulating agent is an increased "alertness" of the immune system meaning that simultaneous or subsequent immunization with an immunogen induces a significantly more effective immune response compared to isolated use of the immunogen.

10 Characteristics of the immunogenic analogues of the invention

The polymeric proteins that are the targets of the presently disclosed strategies may be both homopolymers and heteropolymers. As will be clear from the examples, the most important feature in the first aspect of the invention is that the polymeric protein in question can be "monomerized" without introducing significant changes in the 3 dimensional structure of the multimeric protein. Hence, the particular function of the multimeric protein is not important for the gist of the present invention - rather it is the structural characteristics of the protein that decides whether or not it is a suitable candidate for the present approach in the first aspect of the invention. For instance, if the N-terminus of one monomer in the multimeric protein has a spatial proximity to the C-terminus of another monomer in the multimer, the linking of these two particular monomers via a peptide linker may be accomplished without imposing significant changes relative to the structure of the native multimeric protein. If, on the other hand, the termini are far apart, the practice of the present invention requires that large parts of at least one of the monomers is irrelevant for the immunogenic purpose of the invention or that linking between monomeric subunits can be done

with a long linker peptide without this having a negative impact on the antigenic characteristics of the protein.

In the second aspect of the invention, the "immunogenization" of the self-protein monomer unit is made in such a way, that
5 the resulting variant monomer is still capable of forming part of a polymer protein that shares the quarternary structure of the native polymeric self-protein.

It is advantageous if the immunogenic analogue according to the invention displays, in the substantial fragments, a substantial fraction of B-cell epitopes found in the corresponding monomers when being part of the polymeric protein. A substantial fraction of B-cell epitopes is herein intended to mean a fraction of B-cell epitopes that antigenically characterises the multimeric protein versus other proteins. It is
15 preferred that the substantial fragments display essentially all B-cell epitopes found in the corresponding monomers when being part of the polymeric protein - of course, introduction of minor changes in the monomer sequence may be necessary. For instance an amino acid sequence derived from a monomeric unit
20 may be modified by means of amino acid insertion, substitution, deletion or addition so as to reduce toxicity of the analogue as compared to the multimeric protein and/or so as to introduce the MHC Class II binding amino acid sequence, if it is undesired to have that sequence positioned in a linker.

25 An especially preferred embodiment provides for an immunogenic analogue of the invention, wherein each of the substantial fractions comprises essentially the complete amino acid sequence of each monomeric unit, either as a continuous sequence or as a sequence including inserts. That is, only insignificant parts of the monomeric unit's sequence are left out of
30

the analogue, e.g. in cases where such a sequence does not contribute to tertiary structure of the monomeric unit or quaternary structure of the multimeric protein. However, this embodiment allows for substitution or insertion of the monomer, as long as the 3D structure of the multimeric protein is maintained. Hence, it is especially advantageous if the immunogenic analogue is one, wherein amino acid sequences of all monomeric units of the polymeric proteins are represented in the analogue, and it is particularly advantageous if the analogue includes the complete amino acid sequences of (all) the monomers constituting the polymeric protein, either as unbroken sequences or as sequences including inserts.

As will appear, it is therefore preferred that the 3-dimensional structure of the complete polymeric protein is essentially preserved in the analogue.

Demonstration of maintenance of a substantial fraction of B-cell epitopes or even the 3-dimensional structure of a multimeric protein that is subjected to modification as described herein can be achieved in several ways. One is simply to prepare a polyclonal antiserum directed against the multimer (e.g. an antiserum prepared in a rabbit) and thereafter use this antiserum as a test reagent (e.g. in a competitive ELISA) against the modified proteins which are produced. Modified versions (analogues) which react to the same extent with the antiserum as does the multimer must be regarded as having the same 3D structure as the multimer whereas analogues exhibiting a limited (but still significant and specific) reactivity with such an antiserum are regarded as having maintained a substantial fraction of the original B-cell epitopes.

Alternatively, a selection of monoclonal antibodies reactive with distinct epitopes on the multimer can be prepared and used as a test panel. This approach has the advantage of allowing 1) an epitope mapping of the multimer and 2) a mapping of the epitopes which are maintained in the analogues prepared.

Of course, a third approach would be to resolve the 3-dimensional structure of the multimer (cf. above) and compare this to the resolved three-dimensional structure of the analogues prepared. Three-dimensional structure can be resolved by the aid of X-ray diffraction studies and NMR-spectroscopy. Further information relating to the tertiary structure can to some extent be obtained from circular dichroism studies which have the advantage of merely requiring the polypeptide in pure form (whereas X-ray diffraction requires the provision of crystallized polypeptide and NMR requires the provision of isotopic variants of the polypeptide) in order to provide useful information about the tertiary structure of a given molecule. However, ultimately X-ray diffraction and/or NMR are necessary to obtain conclusive data since circular dichroism can only provide indirect evidence of correct 3-dimensional structure via information of secondary structure elements.

The immunogenic analogue of the invention may include a peptide linker that includes or contributes to the presence in the analogue of at least one MHC Class II binding amino acid sequence that is heterologous to the multimeric protein. This is particularly useful in those cases where it is undesired to alter the amino acid sequence corresponding to monomeric units in the multimeric protein. Alternatively, the peptide linker may be free of and not contributing to the presence of an MHC Class II binding amino acid sequence in the animal species

from where the multimeric protein is derived; this can conveniently be done in cases where it is necessary to utilise a very short linker or where it is advantageous to e.g. detoxify a potentially toxic analogue by introducing the MHC Class II binding element in an active site. Both these embodiments can be combined with introduction of point mutations that detoxify the molecule if need be.

It is preferred that the MHC Class II binding amino acid sequence binds a majority of MHC Class II molecules from the animal species from where the multimeric protein has been derived, i.e. that the MHC Class II binding amino acid sequence is universal or promiscuous.

It is of course important that this sequence serves its purpose as a T cell epitope in the species for which the immunogen is intended to serve as a vaccine constituent. There exists a number of naturally occurring "promiscuous" T-cell epitopes which are active in a large proportion of individuals of an animal species or an animal population and these are preferably introduced in the vaccine, thereby reducing the need for a very large number of different analogues in the same vaccine. Hence, the at least one MHC Class II binding amino acid sequence is preferably selected from a natural T-cell epitope and an artificial MHC-II binding peptide sequence. Especially preferred sequences are a natural T-cell epitope is selected from a Tetanus toxoid epitope such as P2 (SEQ ID NO: 3) or P30 (SEQ ID NO: 5), a diphtheria toxoid epitope, an influenza virus hemagglutinin epitope, and a *P. falciparum* CS epitope.

Over the years a number of other promiscuous T-cell epitopes have been identified. Especially peptides capable of binding a

large proportion of HLA-DR molecules encoded by the different HLA-DR alleles have been identified and these are all possible T-cell epitopes to be introduced in the analogues used according to the present invention. Cf. also the epitopes discussed
5 in the following references which are hereby all incorporated by reference herein: WO 98/23635 (Frazer IH et al., assigned to The University of Queensland); Southwood S et al., 1998, J. Immunol. **160**: 3363-3373; Sinigaglia F et al., 1988, Nature **336**: 778-780; Chicz RM et al., 1993, J. Exp. Med **178**: 27-47;
10 Hammer J et al., 1993, Cell **74**: 197-203; and Falk K et al., 1994, Immunogenetics **39**: 230-242. The latter reference also deals with HLA-DQ and -DP ligands. All epitopes listed in these 5 references are relevant as candidate natural epitopes to be used in the present invention, as are epitopes that
15 share common motifs with these.

Alternatively, the epitope can be any artificial T-cell epitope which is capable of binding a large proportion of MHC Class II molecules. In this context the pan DR epitope peptides ("PADRE") described in WO 95/07707 and in the corresponding paper Alexander J et al., 1994, Immunity **1**: 751-761
20 (both disclosures are incorporated by reference herein) are interesting candidates for epitopes to be used according to the present invention. It should be noted that the most effective PADRE peptides disclosed in these papers carry D-amino
25 acids in the C- and N-termini in order to improve stability when administered. However, the present invention primarily aims at incorporating the relevant epitopes as part of the analogue which should then subsequently be broken down enzymatically inside the lysosomal compartment of APCs to allow
30 subsequent presentation in the context of an MHC-II molecule and therefore it is not expedient to incorporate D-amino acids in the epitopes used in the present invention.

- One especially preferred PADRE peptide is the one having the amino acid sequence AKFVAAWTLKAAA (SEQ ID NO: 7) or an immunologically effective subsequence thereof. This, and other epitopes having the same lack of MHC restriction are preferred
- 5 T-cell epitopes which should be present in the analogues used in the inventive method. Such super-promiscuous epitopes will allow for the most simple embodiments of the invention wherein only one single modified IL5 is presented to the vaccinated animal's immune system.
- 10 Preferred embodiments of the invention includes modification by introducing at least one foreign immunodominant T_H epitope. It will be understood that the question of immune dominance of a T_H epitope depends on the animal species in question. As used herein, the term "immunodominance" simply refers to epitopes
- 15 which in the vaccinated individual gives rise to a significant immune response, but it is a well-known fact that a T_H epitope which is immunodominant in one individual is not necessarily immunodominant in another individual of the same species, even though it may be capable of binding MHC-II molecules in the
- 20 latter individual.

As mentioned above, the introduction of a foreign T-cell epitope can be accomplished by introduction of at least one amino acid insertion, addition, deletion, or substitution. Of course, the normal situation will be the introduction of more

25 than one change in the amino acid sequence (e.g. insertion of or substitution by a complete T-cell epitope) but the important goal to reach is that the analogue, when processed by an antigen presenting cell (APC), will give rise to such a T-cell epitope being presented in context of an MCH Class II molecule

30 on the surface of the APC. Thus, if the amino acid sequence of the monomeric unit in appropriate positions comprises a number

of amino acid residues which can also be found in a foreign T_H epitope then the introduction of a foreign T_H epitope can be accomplished by providing the remaining amino acids of the foreign epitope by means of amino acid insertion, addition, 5 deletion and substitution. In other words, it is not necessary to introduce a complete T_H epitope by insertion or substitution.

According to the present invention, the analogue may also form part of larger molecule wherein it is coupled to at least one 10 functional moiety, the presence of which does not interfere negatively to a significant degree with the antibody-accessability of the analogue. The nature of such moieties (which may be fused to the analogue) can be to target the modified molecule to an antigen presenting cell (APC) or a B- 15 lymphocyte, to stimulate the immune system, and to optimize presentation of the analogue to the immune system.

Targeting moieties are conveniently selected from the group consisting of a substantially specific binding partner for a B-lymphocyte specific surface antigen or for an APC specific 20 surface antigen, such as a hapten or a carbohydrate for which there is a receptor on the B-lymphocyte or the APC. The immunestimulating moieties may be selected from the group consisting of a cytokine, a hormone, and a heat-shock protein. The presentation optimising moiety may be selected from the 25 group consisting of a lipid group, such as a palmitoyl group, a myristyl group, a farnesyl group, a geranyl-geranyl group, a GPI-anchor, and an N-acyl diglyceride group.

A suitable cytokine is, or is an effective part of any of, interferon γ (IFN- γ), Flt3L, interleukin 1 (IL-1), interleukin 2 30 (IL-2), interleukin 4 (IL-4), interleukin 6 (IL-6), inter-

leukin 12 (IL-12), interleukin 13 (IL-13), interleukin 15 (IL-15), and granulocyte-macrophage colony stimulating factor (GM-CSF).

A preferred heat-shock protein is, or is an effective part of
5 any of, HSP70, HSP90, HSC70, GRP94, and calreticulin (CRT).

It is preferred that the number of amino acid insertions, deletions, substitutions or additions is at least 2, such as 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, and 25 insertions, substitutions, additions or deletions.
10 It is furthermore preferred that the number of amino acid insertions, substitutions, additions or deletions is not in excess of 150, such as at most 100, at most 90, at most 80, and at most 70. It is especially preferred that the number of substitutions, insertions, deletions, or additions does not ex-
15 ceed 60, and in particular the number should not exceed 50 or even 40. Most preferred is a number of not more than 30. With respect to amino acid additions, it should be noted that these, when the resulting construct is in the form of a fusion polypeptide, is often considerably higher than 150.

20 Preferred embodiments of the invention includes modification by introducing at least one foreign immunodominant T_H epitope (= "foreign MHC Class II binding amino acid sequence"). It will be understood that the question of immune dominance of a T_H epitope depends on the animal species in question. As used
25 herein, the term "immunodominance" simply refers to epitopes which in the vaccinated individual gives rise to a significant immune response, but it is a well-known fact that a T_H epitope which is immunodominant in one individual is not necessarily immunodominant in another individual of the same species, even

though it may be capable of binding MHC-II molecules in the latter individual.

Another important point is the issue of MHC restriction of T_H epitopes. In general, naturally occurring T_H epitopes are MHC restricted, i.e. a certain peptide constituting a T_H epitope will only bind effectively to a subset of MHC Class II molecules. This in turn has the effect that in most cases the use of one specific T_H epitope will result in a vaccine component which is effective in a fraction of the population only, and depending on the size of that fraction, it can be necessary to include more T_H epitopes in the same molecule, or alternatively prepare a multi-component vaccine wherein the components are variants which are distinguished from each other by the nature of the T_H epitope introduced.

15 If the MHC restriction of the T-cells used is completely unknown (for instance in a situation where the vaccinated animal has a poorly defined MHC composition), the fraction of the animal population covered by a specific vaccine composition can be determined by means of the following formula:

$$20 \quad f_{\text{population}} = 1 - \prod_{i=1}^n (1 - p_i) \quad (\text{II})$$

-where p_i is the frequency in the population of responders to the i^{th} foreign T-cell epitope present in the vaccine composition, and n is the total number of foreign T-cell epitopes in the vaccine composition. Thus, a vaccine composition containing 3 foreign T-cell epitopes having response frequencies in the population of 0.8, 0.7, and 0.6, respectively, would give

$$1 - 0.2 \times 0.3 \times 0.4 = 0.976$$

-i.e. 97.6 percent of the population will statistically mount an MHC-II mediated response to the vaccine.

The above formula does not apply in situations where a more or less precise MHC restriction pattern of the peptides used is known. If, for instance a certain peptide only binds the human MHC-II molecules encoded by HLA-DR alleles DR1, DR3, DR5, and DR7, then the use of this peptide together with another peptide which binds the remaining MHC-II molecules encoded by HLA-DR alleles will accomplish 100% coverage in the population in question. Likewise, if the second peptide only binds DR3 and DR5, the addition of this peptide will not increase the coverage at all. If one bases the calculation of population response purely on MHC restriction of T-cell epitopes in the vaccine, the fraction of the population covered by a specific vaccine composition can be determined by means of the following formula:

$$f_{population} = 1 - \prod_{j=1}^3 (1 - \phi_j)^2 \quad (III)$$

-wherein ϕ_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind any one of the T-cell epitopes in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ); in practice, it is first determined which MHC molecules will recognize each T-cell epitope in the vaccine and thereafter these MHC molecules are listed by type (DP, DR and DQ) - then, the individual frequencies of the different listed allelic haplotypes are summed for each type, thereby yielding ϕ_1 , ϕ_2 , and ϕ_3 .

It may occur that the value p_i in formula II exceeds the corresponding theoretical value π_i :

$$\pi_i = 1 - \prod_{j=1}^3 (1 - v_j)^2 \quad (\text{IV})$$

-wherein v_j is the sum of frequencies in the population of allelic haplotypes encoding MHC molecules which bind the i^{th} T-cell epitope in the vaccine and which belong to the j^{th} of the 3 known HLA loci (DP, DR and DQ). This means that in $1 - \pi_i$ of the population there is a frequency of responders of $f_{\text{residual}_i} = (p_i - \pi_i) / (1 - \pi_i)$. Therefore, formula III can be adjusted so as to yield formula V:

$$f_{\text{population}} = 1 - \prod_{j=1}^3 (1 - \varphi_j)^2 + \left(1 - \prod_{i=1}^n (1 - f_{\text{residual}_i}) \right) \quad (\text{V})$$

10 -where the term $1 - f_{\text{residual}_i}$ is set to zero if negative. It should be noted that formula V requires that all epitopes have been haplotype mapped against identical sets of haplotypes.

Therefore, when selecting T-cell epitopes to be introduced in the analogue of the invention, it is important to include all knowledge of the epitopes which is available: 1) The frequency of responders in the population to each epitope, 2) MHC restriction data, and 3) frequency in the population of the relevant haplotypes.

It should be noted that preferred analogues of the invention 20 comprise modifications which results in a polypeptide that includes stretches having a sequence identity of at least 70% with the corresponding monomeric units of the multimeric protein or with subsequences thereof of at least 10 amino acids in length. Higher sequence identities are preferred, e.g. at least 75% or even at least 80% or 85%. The sequence identity 25 for proteins and nucleic acids can be calculated as $(N_{\text{ref}} - N_{\text{dif}}) \cdot 100 / N_{\text{ref}}$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Hence, the DNA

sequence AGTCAGTC will have a sequence identity of 75% with the sequence AATCAATC ($N_{dif}=2$ and $N_{ref}=8$).

Finally, in order to conclusively verify that an analogue of the invention is indeed effective as an immunogen, various tests may be performed in order to provide the necessary confirmation, cf. also the specifics set forth in the examples herein. In this context, reference is also made to the discussion of identification of useful IL5 analogues in WO 00/65058 - this disclosure may be used for verification of the usefulness of an analogue (IL5 derived or not) subject to the present inventive technology.

Preferred multimers that may be subjected to the technology of the present invention are IL5 and TNF α .

IL5 based constructs

For hIL5 it has been found that constructs that mimic the natural hIL5 dimer structure and at the same time include foreign T_H elements provide superior results compared to constructs based on the monomeric structure, e.g. over the constructs disclosed in WO 00/65058, especially when it comes to expression levels and antibody reactivity of antisera raised against the constructs.

Preferred constructs based on IL5 are those wherein the analogue is selected from the group consisting of

- two complete IL5 monomers joined by a peptide linker that includes at least one MHC Class II binding amino acid sequence, and

- two complete IL5 monomers joined by an inert peptide linker of which at least one IL5 monomer includes a heterologous MHC Class II binding amino acid sequence.

Such an analogue may have the linear structure IL-L_m-IL or IL_m-L_i-IL_n or IL-L_i-IL_m or IL-L_i-IL_m or IL_m-L_m-IL_n wherein "IL" is the complete amino acid sequence of monomeric mature IL5, "IL_m" and "IL_n", which may be identical or non-identical, designate a substantially complete amino acid sequence of monomeric mature IL5 including a heterologous MHC Class II binding amino acid sequence, "L_m" is a peptide linker including or contributing to at least one MHC Class II binding amino acid sequence in the analogue, and "L_i" is an inert peptide linker that does not include or contribute to any MHC Class II binding amino acid sequence in the analogue. It is especially preferred that L_m, IL_m and IL_n comprise the P2 and/or P30 epitopes of tetanus toxoid or comprises a PADRE, and L_i is a di-glycine linker. However L_i may be any non-immunogenic linker peptide that does not give rise to MHC Class II binding sequences.

Most preferred embodiments are hIL5 analogues having the mature amino acid sequence set forth in any one of SEQ ID NOS: 9, 11, 13 and 15.

TNFα background

Tumour necrosis factor (TNF, TNFα, cachectin, TNFSF2) is a potent paracrine and endocrine mediator of inflammatory and immune functions. TNFα is cytotoxic for many cells especially in combination with gamma-interferon. TNFα was initially identified in 1975 and demonstrated to initiate tumor necrosis and regression. The anti-cancer effect has later been investigated in detail, but the treatment has not been a success as cancer

therapy, although there are still cancer trials using TNF α running. TNF α was later discovered as the cause of cachexia and it was discovered that TNF exerts its function through a receptor-mediated process. Two different TNF α receptors
5 (TNFR55 and TNFR75) have been identified that mediate cytotoxic and inflammatory effects of TNF α . TNF α induces and perpetuates inflammatory processes during chronic inflammatory diseases like rheumatoid arthritis (RA) and is suspected to have a critical role in allergies and psoriasis. Blocking of
10 the TNF α signal by soluble receptors, receptor-specific inhibitors, down-regulation of TNF α production or monoclonal anti-TNF α antibodies are attractive therapy forms to adverse the biological effects of TNF α up-regulation and signaling.

It is evident from the results obtained from treatment with
15 soluble TNF α receptors and monoclonal anti-TNF α antibodies that anti-TNF α therapy is a success in several diseases, like RA and Crohn's disease. The anti-TNF α treatment is both considered safe and effective.

To date, two TNF α antagonists, Remicade (Infliximab, Cento-
20 cor/Johnson&Johnson) and Enbrel (Etanercept, Immunex) have been approved for clinical use.

Remicade is a chimeric mouse-human monoclonal IgG1 antibody directed against soluble and cell associated TNF α . Remicade blocks the binding of TNF with its endogenous cell surface
25 TNF α receptor. The Food and Drug Administration (FDA) approved Remicade in October 1998 for use in moderate to severe or fistulizing Crohn's Disease refractory to conventional therapies. The indication was extended to include adjunctive use with methotrexate in rheumatoid arthritis refractory to methotrex-

ate therapy alone and in July 2002 maintenance therapy in Crohn's disease.

Enbrel is a recombinant protein consisting of the extracellular portion of the human TNF α receptor fused to the Fc portion of human IgG1. Enbrel inhibits TNF α activity by serving as a decoy TNF α receptor. FDA approved Enbrel for use in rheumatoid arthritis in November 1998. More than 350,000 patients have been treated with these TNF α antagonists. Review of clinical efficacy and safety information of these agents are performed continuously and although infections and other immune-related adverse events remain a major concern for TNF α antagonists, recent safety evaluation of post-marketing experience performed by the FDA and the Committee for Proprietary Medicinal Products (CPMP) states that anti-TNF α therapies have a favorable risk-benefit balance although labelling changes, including changes on serious infections have been required.

Compared with the established anti-TNF α therapies, the presently suggested TNF α immunotherapy has the advantages of microgram amount vaccinations and less frequent injections to keep a high anti-TNF α *in vivo* titer compared with large infusions of monoclonal antibodies. The positive consequences are a lower risk for side effects and less expensive therapy. It is also believed that a natural polyclonal antibody response will act as a more efficient down-regulator of TNF α than other anti-TNF α therapies.

TNF α is translated as a 233 amino acid precursor protein and secreted as a trimeric type II transmembrane protein, which is cleaved by specific metalloproteases to a trimeric soluble protein where each identical monomeric subunit consists of 157

amino acids (the amino acid sequence of which is set forth in SEQ ID NO: 17). Human TNF α is non-glycosylated while murine TNF α has a single N-glycosylation site. The TNF α monomer has a molecular weight of 17 kDa while the trimer has a theoretical MW of 52 kDa, although a cross-linked trimer moves as 43 kDa in SDS-PAGE. TNF α contains two cysteines that stabilize the structure by forming an intramolecular disulphide bridge. Both the N and C-terminus of TNF α are important for the activity. Especially the C-terminus is sensitive as deletion of three, two and even one amino acid drastically decreases the solubility and bioactivity. The important amino acid is Leu157, which forms a stabilizing salt bridge between two monomers in the trimer. On the other hand deletion of the first eight amino acids increases the activity with a factor 1.5-5 while deletion of the first nine amino acids restores the full-length activity. TNF α is a well-studied protein and many of the intra- and inter-molecular interactions leading to trimer formation and receptor binding have been identified.

Hence, in nature, human TNF α (SEQ ID NO: 17) exists as both a dimer and a trimer, but the molecule is in both cases very suitable as a candidate target for the present invention.

TNF α constructs

A preferred TNF α analogue is selected from the group consisting of 1) two or three complete TNF- α monomers joined end-to-end by a peptide linker, wherein at least one peptide linker includes at least one MHC Class II binding amino acid sequence, and 2) two or three complete TNF- α monomers joined end-to-end by an inert peptide linker, wherein at least one of the monomers include at least one foreign MHC Class II binding

amino acid sequence or wherein at least one foreign MHC Class II binding amino acids sequence is fused to the N- or C-terminal monomer, optionally via an inert linker.

Particularly interesting are immunogenic TNF α molecules with high stability, since it has earlier been found by the inventors that monomeric TNF α constructs tend to be relatively unstable, cf., however, the discussion below.

Thus, this type of construct is very much in analogy to the above discussed IL5 constructs.

10 A gene encoding the 3 TNF α subunits linked together by epitopes and/or inert peptide linkers in a manner parallel to that discussed for IL5 has been produced. The goal has been to generate variant TNF α molecules with a conformation as close to the native TNF α trimer as possible. The variants are designed
15 to efficiently elicit neutralizing antibodies against wtTNF α . The most suitable TNF α variants are soluble and stable proteins in the absence of detergents or other kinds of additives that could disrupt the protein conformation.

By expressing the three monomers linked together as one single
20 polypeptide chain using linkers and T_H epitopes, it is intended to prepare TNF α variants that are more stable than previous variant TNF α immunogens. This will allow preservation of the TNF α structure, by introduction of the necessary T_H epitopes outside of stabilizing hydrogen bonds, salt bridges or disul-
25 fide bridges.

From the X-ray crystal structure of TNF α it is seen that the first 5 residues of the N terminal are too flexible to allow a

structure determination. The C-terminus, however, is located close to the middle of the monomer interface and is less flexible. The distance between the C alpha atoms of Arg-6 and Leu-157 is 10 Å, which is the distance of 3-4 amino acid residues. Therefore it seems to be possible to link the monomeric subunits directly together, but since the C-terminals are located at a delicate site, it will probably be advantageous to use flexible linkers, e.g. glycine linkers, for this connection.

10 Five variants have until now been designed utilising the "monomerized trimer" approach. The control TNF_T0 (TNF α Trimer number 0, SEQ ID NO: 22) consists of the three monomers directly linked together by 2 separate glycine linkers (GlyGlyGly). TNF_T0 is designed so as to be as stable as the
15 wild type trimeric protein. Of course, other inert flexible linkers known in the art of protein chemistry may be used instead of the above-mentioned glycine linkers, the important feature being that the flexible linker does not interfere adversely with the monomerized protein's capability of folding
20 into a 3D structure that is similar to the 3D structure of physiologically active wtTNF α .

The TNF_T0 construct is expressed as a soluble protein in *E. coli*, and it has been used to prepare the exemplary construct TNF_T4 (SEQ ID NO: 57), which is a variant wherein the PADRE
25 MHC Class II binding peptide (SEQ ID NO: 7) is introduced. In this construct, the ratio between monomeric units and foreign epitopes are thus 1 epitope per 3 monomers, instead of 1 epitope per monomer as is the case in prior art variants that relied on immunogenized monomeric proteins - this is also the
30 case for SEQ ID NO: 55). This fact provides a potentially positive influence on the trimer stability. An offspring from

this approach is the TNF_C2 variant (SEQ ID NO: 28, cf. below), which is a TNF α monomer with a PADRE epitope in the same position as in TNF_T4.

In parallel, the tetanus toxoid P2 and P30 epitopes (SEQ ID NOs: 3 and 5, respectively), have been used in the TNF_T1 and TNF_T2 variants (SEQ ID NOs: 49 and 51, respectively), containing one epitope in each linker region, and also in TNF_T3 (SEQ ID NO: 59) that contains one C-terminal epitope and one in the second linker region. Proteins are mostly folded from the N-terminal toward the C-terminal. The idea underlying TNF_T3 is that when the first two N-terminal domains fold up they will function as internal chaperones for the third domain (monomer), which is enclosed by epitopes.

It has been discovered that in addition to the technology described in detail above, where polymeric proteins are "monomerized", TNF α (and possibly many other multimeric proteins) allows for the production of monomers that 1) include at least one stabilising mutation and/or 2) include at least one non-TNF α derived MHC Class II binding amino acid sequence, where these TNF α monomer variants are capable of folding correctly into a tertiary structure that subsequently allows for the formation of dimeric and trimeric TNF α proteins having a correct quaternary structure (as evidenced by these having receptor binding activity). Hence, in these constructs it has been possible to prepare variants of monomeric TNF α that does not necessarily need to be produced as monomerized trimers because the changes introduced in the monomer sequences introduce so limited disruption of the monomer's tertiary structure that a di- or trimer can be formed. In accordance with the ideas underlying the present invention, it has

further been found that all such variant are expressible as soluble proteins from bacterial cells.

Hence, it is possible to prepare immunogenic TNF α variants according to the following strategies that can be combined and
5 which may further be combined with the already discussed "monomerization approach" of the invention (since these particular modifications alle are non-destructive by nature):

The flexible loop strategy

It has been discovered by the present inventors that insertion
10 of the PADRE epitope (SEQ ID NO: 7) into loop 3 in position Gly108-Ala109 is a promising approach to prepare TNF α variants with a structure closely resembling the native TNF α molecule. It has been deduced from the TNF α crystal structure that a T_H epitope inserted directly into this position will not have any
15 neighboring amino acid residues in close proximity to interact with. Studies with TNF34 (SEQ ID NO: 18), the first PADRE construct made according to this approach, has shown that approximately 5% of the expressed protein TNF34 was soluble in E. coli and 95% of the TNF34 was expressed as inclusion bodies
20 when the bacterial host cells were grown at 37°C but after an adaptation of the fermentation process where the fermentation temperature is 25°C, the yields of soluble protein from the fermentation is close to 100%. Hence, optimization of growth conditions increases the yield of soluble protein.

25 A number of other constructs have been prepared (TNF35-TNF39, SEQ ID NOs, 23, 24, 25, 26, and 27), where all of these solely rely on introduction of SEQ ID NO: 7 in the flexible loop 3.

Stability enhancing mutations

Introduction of T_H epitopes in the flexible loop 3 could potentially destabilize the structure of the TNF α variant. However, this potential destabilization can be counteracted by stabilization of the structure through introduction of cysteines that will form a disulfide bridge. A cystine pair in two different positions have until now been introduced in variants TNF34-A and TNF34-B (SEQ ID NOs: 29 and 30). Also, the flexible N-terminal (the first 8 amino acids) that is known to reduce the strength of the receptor interaction will be deleted in parallel, hence the variant TNF34-C (SEQ ID NO: 31). The disulfide bridge is introduced in the monomer for stabilization of the epitope insertion site together with the naturally occurring disulfide bridge (Cys-67 Cys-101). This strategy would also stabilize both a TNF α monomer as such and a monomerized di- or trimer.

Other conststructs

Several different strategies have been employed in the design of variants that will be soluble expression products. TNFX1.1-2 (SEQ ID NOs: 32 and 33) are based on insertions of SEQ ID NO: 7 in the first loop of TNF α , where the insertion site is located at an intron position. In TNFX2.1 (SEQ ID NO: 34) an artificial "stalk" region is created containing an insertion of SEQ ID NO: 7.

Mutations of TNF α have revealed that large hydrophobic amino acid substitutions, pointing into the trimer interface, stabilize the trimer structure. TNFX3.1 and TNFX3.2 (SEQ ID NOs: 35 and 36) are proposals to stabilize the existing TNF34 variant. TNFX4.1 (SEQ ID NO: 37) uses di-glycine linkers to diminish

structural constraints from the PADRE peptide on the overall TNF34 structure. TNFX5.1 (SEQ ID NO: 38) employs, as an insertion point, a loop structure found in the TNF family member BlyS. TNFX6.1-2, TNF7.1-2 and TNFX8.1 (SEQ ID NOs: 39, 40, 41, 5 42, and 43) are further variants. TNFX9.1 and TNFX9.2 (SEQ ID NOs: 44 and 45) are TNF34 variants that utilize identical overlapping TNF α sequences of 4-6 amino acids both pre and post the epitope. Finally, two variants (SEQ ID NOs: 46 and 47) are P2/P30 double variants in the same location as for the 10 PADRE peptide in TNF34.

Further, from the crystal structure of TNF α it is observed that one stabilizing salt bridge is present within the TNF α monomer between the residues Lys-98 and Glu-116. The definition of a salt-bridge is an electrostatic interaction between 15 side chain oxygens in Asp or Glu and positive charged atom side chain nitrogens in Arg, Lys or His with an interatomic distance less than 7.0 Ångstrom. By site directed substitution mutations of Lys-98 with Arg or His at this position in combination with substitutions of Glu 116 with Asp, an improvement 20 of the stability for this salt bridge and thereby the stability of the trimer molecule could be attained. It is also possible to exchange these salt bridges with disulphide bridges, in a manner described above.

It has been observed that murine TNF α is considerably more 25 stable than the human TNF α regarding to solubility and proteolysis. Improvement of TNF α variants includes making site directed mutants so as to mimic murine TNF α crystal structure to obtain more proteolytically stable TNF α product.

From the x-ray structures of human and murine TNF α it is seen that the centre of the trimer (in the middle of the three TNF α monomers) is held together due to hydrophobic forces, whereas the top and the bottom of the trimer is connected due to natural occurring salt bridges. Therefore, by screening these salt bridges for stronger connections, the stability of the TNF α trimer would also be improved.

Finally, the preliminary results obtained with the TNF α variants of the present invention have surprisingly demonstrated that the variants are physiologically active, at least in the sense that they bind the TNF-receptors. However, since TNF α is a toxic protein, it is desired to prepare safe variants that will not cause severe side effects in subjects immunised with a vaccine according to the invention. Therefore, it is also an important embodiment of the invention to include detoxifying mutations in the constructs if these upon testing in relevant toxicity models are demonstrated to be of potential danger for vaccinated individuals.

A number of point mutations are known in the art to detoxify TNF α or at least reduce toxicity to a large extent. These point mutations will, if necessary, be introduced into the variants of the present invention. Especially preferred mutations are substitutions corresponding to mature TNF α of Tyr-87 with a Ser, of Asp-143 with Asn, and of Ala-145 with Arg. Further, all effective mutations mentioned in Loetscher, H., Stueber, D., Banner, D., Mackay, F. and Lesslauer, W. 1993 JBC 268 (35) 26350-7, are also interesting embodiments in the detoxifying embodiments of the present invention.

In summary, the following specific TNF α variants have been prepared according to the present invention:

TNF Constructs	Last aa before epitope	First aa after epitope	Amino acids deleted by insert	Mutations	Total length
TNF34	108	109	-		170
TNF35	106	107	-		170
TNF36	107	108	-		170
TNF37	108	110	A		169
TNF38	108	112	AEA		167
TNF39	106	112	EGAEA		165
TNFC2	170		-	GGG+PADRE added C-terminally	173
TNF34-A	108	109	-	Q67C, A111C	170
TNF34-B	108	109	-	A96C, I118C	170
TNF34-C	108	109	-	N-terminal VRSSSRTP are deleted	162
TNFX1.1	17	19	A		169
TNFX1.2	17	96	ANPQA		165
TNFX2.1	0	2	V	PADRE added N-terminally	170
TNFX3.1	108	109	-	L157F	170
TNFX3.2	108	109	-	V49F	170
TNFX4.1	108	109	-	Two glycines before and after PADRE	174
TNFX5.1	83	87	AVS		167
TNFX6.1	132	146	SAEINRPDYLDFA		157
TNFX6.2	135	146	INRPDYLDFA		160
TNFX7.1	63	77	FKGQGCPSTHVLL		157
TNFX7.2	71	85	THVLLTHTISRIA		157
TNFX8.1	126	140	EKGDRLSAEINRP		157
TNFX9.1	108	103	-	The six amino acids preceeding PADRE are duplicated after the epitope	176
TNFX9.2	108	105	-	The four amino acids preceeding PADRE are duplicated after the epitope	174
TNF34-P2-P30	108	109	-	Both P2 and P30	194
TNF34-P30-P2	108	109	-	Both P30 and P2	194

The numbers used are from the N-terminal V in SEQ ID NO: 17
5 (that is, from amino acid no. 2 in SEQ ID NO: 17). Preceeding

the N-terminal Valine is in some sequences a Methionine used for translation start.

The most preferred protein constructs of the invention are thus those represented by any one of SEQ ID NOs: 18, 23, 24,
5 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 49, 51, 53, 55, 57, and 59, as well as any amino acid sequence derived therefrom that only include conservative amino acid changes or detoxifying amino acid changes thereof.

10 At any rate, it is an important embodiment that all of these TNF α variants discussed above are expressible as soluble proteins from bacterial cells such as *E. coli*.

The preferred vector is pET28b+ when the goal is expression from *E. coli*, pZop2F (SEQ ID NO: 60) is the vector used for
15 insect cell expression, and pHP1 (or its commercially available "twin" pCI) is the vector used for expression in mammalian cells

General therapies provided by the invention

The invention provides for methods whereby it becomes possible
20 to down-regulate a particular polymeric protein in a very advantageous manner.

In general, there is provided a method for down-regulating a polymeric protein in an autologous host, the method comprising effecting presentation to the animal's immune system of an im-
25 munogenically effective amount of at least one immunogenic analogue of the invention. It is preferred that the autologous host is a mammal, most preferably a human being.

The method can be put into practice in a number of ways, of which administration of a protein vaccine is one choice, but also a nucleic acid vaccination strategy or a live vaccination strategy are of great interest.

5 Protein/polypeptide vaccination and formulation

When effecting presentation of the analogues to an animal's immune system by means of administration thereof to the animal, the formulation of the polypeptide follows the principles generally acknowledged in the art.

- 10 Preparation of vaccines which contain peptide sequences as active ingredients is generally well understood in the art, as exemplified by U.S. Patents 4,608,251; 4,601,903; 4,599,231; 4,599,230; 4,596,792; and 4,578,770, all incorporated herein by reference. Typically, such vaccines are prepared as in-
- 15 jectables either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in, liquid prior to injection may also be prepared. The preparation may also be emulsified. The active immunogenic ingredient is often mixed with excipients which are pharmaceutically acceptable and com-
- 20 patible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vaccine may contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pH buffering agents, or
- 25 adjuvants which enhance the effectiveness of the vaccines; cf. the detailed discussion of adjuvants below.

The vaccines are conventionally administered parenterally, by injection, for example, either subcutaneously, intracutaneously, intradermally, subdermally or intramuscularly. Additional formulations which are suitable for other modes of ad-

30

ministration include suppositories and, in some cases, oral, buccal, sublingual, intraperitoneal, intravaginal, anal, epidural, spinal, and intracranial formulations. For suppositories, traditional binders and carriers may include, for example, polyalkylene glycols or triglycerides; such suppositories may be formed from mixtures containing the active ingredient in the range of 0.5% to 10%, preferably 1-2%. Oral formulations include such normally employed excipients as, for example, pharmaceutical grades of mannitol, lactose, starch, magnesium stearate, sodium saccharine, cellulose, magnesium carbonate, and the like. These compositions take the form of solutions, suspensions, tablets, pills, capsules, sustained release formulations or powders and contain 10-95% of active ingredient, preferably 25-70%. For oral formulations, cholera toxin is an interesting formulation partner (and also a possible conjugation partner).

The polypeptides may be formulated into the vaccine as neutral or salt forms. Pharmaceutically acceptable salts include acid addition salts (formed with the free amino groups of the peptide) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed with the free carboxyl groups may also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

The vaccines are administered in a manner compatible with the dosage formulation, and in such amount as will be therapeutically effective and immunogenic. The quantity to be administered depends on the subject to be treated, including, e.g.,

the capacity of the individual's immune system to mount an immune response, and the degree of protection desired. Suitable dosage ranges are of the order of several hundred micrograms active ingredient per vaccination with a preferred range from
5 about 0.1 μg to 2,000 μg (even though higher amounts in the 1-10 mg range are contemplated), such as in the range from about 0.5 μg to 1,000 μg , preferably in the range from 1 μg to 500 μg and especially in the range from about 10 μg to 100 μg .
Suitable regimens for initial administration and booster shots
10 are also variable but are typified by an initial administration followed by subsequent inoculations or other administrations.

The manner of application may be varied widely. Any of the conventional methods for administration of a vaccine are applicable.
15 These include oral application on a solid physiologically acceptable base or in a physiologically acceptable dispersion, parenterally, by injection or the like. The dosage of the vaccine will depend on the route of administration and will vary according to the age of the person to be vaccinated
20 and the formulation of the antigen.

Some of the analogues of the vaccine are sufficiently immunogenic in a vaccine, but for some of the others the immune response will be enhanced if the vaccine further comprises an adjuvant substance.

25 Various methods of achieving adjuvant effect for the vaccine are known. General principles and methods are detailed in "The Theory and Practical Application of Adjuvants", 1995, Duncan E.S. Stewart-Tull (ed.), John Wiley & Sons Ltd, ISBN 0-471-95170-6, and also in "Vaccines: New Generation Immunological
30 Adjuvants", 1995, Gregoriadis G et al. (eds.), Plenum Press,

New York, ISBN 0-306-45283-9, both of which are hereby incorporated by reference herein.

It is especially preferred to use an adjuvant which can be demonstrated to facilitate breaking of the autotolerance to autoantigens; in fact, this is essential in cases where un-
5 modified IL5 is used as the active ingredient in the autovaccine. Non-limiting examples of suitable adjuvants are selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine, and a
10 mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ -inulin; and an encapsulating adjuvant. In general it should be noted that the disclosures above which
15 relate to compounds and agents useful as first, second and third moieties in the analogues also refer *mutatis mutandis* to their use in the adjuvant of a vaccine of the invention.

The application of adjuvants include use of agents such as aluminium hydroxide or phosphate (alum), commonly used as 0.05
20 to 0.1 percent solution in buffered saline, admixture with synthetic polymers of sugars (e.g. Carbopol®) used as 0.25 percent solution, aggregation of the protein in the vaccine by heat treatment with temperatures ranging between 70° to 101°C for 30 second to 2 minute periods respectively and also aggregation by means of cross-linking agents are possible. Aggregation by reactivation with pepsin treated antibodies (Fab fragments) to albumin, mixture with bacterial cells such as *C. parvum* or endotoxins or lipopolysaccharide components of gram-negative bacteria, emulsion in physiologically acceptable oil
30 vehicles such as mannide mono-oleate (Aracel A) or emulsion with 20 percent solution of a perfluorocarbon (Fluosol-DA)

used as a block substitute may also be employed. Admixture with oils such as squalene and IFA is also preferred.

According to the invention DDA (dimethyldioctadecylammonium bromide) is an interesting candidate for an adjuvant as is DNA
5 and γ -inulin, but also Freund's complete and incomplete adjuvants as well as *quillaja* saponins such as QuilA and QS21 are interesting as is RIBI. Further possibilities are monophosphoryl lipid A (MPL), the above mentioned C3 and C3d, and muramyl dipeptide (MDP).

- 10 Liposome formulations are also known to confer adjuvant effects, and therefore liposome adjuvants are preferred according to the invention.

Also immunostimulating complex matrix type (ISCOM® matrix) adjuvants are preferred choices according to the invention, especially since it has been shown that this type of adjuvants
15 are capable of up-regulating MHC Class II expression by APCs. An ISCOM® matrix consists of (optionally fractionated) saponins (triterpenoids) from *Quillaja saponaria*, cholesterol, and phospholipid. When admixed with the immunogenic protein, the
20 resulting particulate formulation is what is known as an ISCOM particle where the saponin constitutes 60-70% w/w, the cholesterol and phospholipid 10-15% w/w, and the protein 10-15% w/w. Details relating to composition and use of immunostimulating complexes can e.g. be found in the above-mentioned text-books
25 dealing with adjuvants, but also Morein B et al., 1995, Clin. Immunother. 3: 461-475 as well as Barr IG and Mitchell GF, 1996, Immunol. and Cell Biol. 74: 8-25 (both incorporated by reference herein) provide useful instructions for the preparation of complete immunostimulating complexes.

Another highly interesting (and thus, preferred) possibility of achieving adjuvant effect is to employ the technique described in Gosselin et al., 1992 (which is hereby incorporated by reference herein). In brief, the presentation of a relevant
5 antigen such as an antigen of the present invention can be enhanced by conjugating the antigen to antibodies (or antigen binding antibody fragments) against the Fcγ receptors on monocytes/macrophages. Especially conjugates between antigen and anti-FcγRI have been demonstrated to enhance immunogenicity for
10 the purposes of vaccination.

Other possibilities involve the use of the targeting and immune modulating substances (*i.a.* cytokines) mentioned in the claims as moieties for the protein constructs. In this connection, also synthetic inducers of cytokines like poly I:C are
15 possibilities.

Suitable mycobacterial derivatives are selected from the group consisting of muramyl dipeptide, complete Freund's adjuvant, RIBI, and a diester of trehalose such as TDM and TDE.

Suitable immune targeting adjuvants are selected from the
20 group consisting of CD40 ligand and CD40 antibodies or specifically binding fragments thereof (cf. the discussion above), mannose, a Fab fragment, and CTLA-4.

Suitable polymer adjuvants are selected from the group consisting of a carbohydrate such as dextran, PEG, starch, mannan, and mannose; a plastic polymer such as; and latex such as
25 latex beads.

Yet another interesting way of modulating an immune response is to include the immunogen (optionally together with adjuvants and pharmaceutically acceptable carriers and vehicles)

in a "virtual lymph node" (VLN) (a proprietary medical device developed by ImmunoTherapy, Inc., 360 Lexington Avenue, New York, NY 10017-6501). The VLN (a thin tubular device) mimics the structure and function of a lymph node. Insertion of a VLN under the skin creates a site of sterile inflammation with an upsurge of cytokines and chemokines. T- and B-cells as well as APCs rapidly respond to the danger signals, home to the inflamed site and accumulate inside the porous matrix of the VLN. It has been shown that the necessary antigen dose required to mount an immune response to an antigen is reduced when using the VLN and that immune protection conferred by vaccination using a VLN surpassed conventional immunization using Ribi as an adjuvant. The technology is *i.a.* described briefly in Gelber C et al., 1998, "Elicitation of Robust Cellular and Humoral Immune Responses to Small Amounts of Antigens Using a Novel Medical Device Designated the Virtual Lymph Node", in: "From the Laboratory to the Clinic, Book of Abstracts, October 12th - 15th 1998, Seascape Resort, Aptos, California".

It is expected that the vaccine should be administered at least once a year, such as at least 1, 2, 3, 4, 5, 6, and 12 times a year. More specifically, 1-12 times per year is expected, such as 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 times a year to an individual in need thereof. It has previously been shown that the memory immunity induced by the use of the preferred autovaccines according to the invention is not permanent, and therefor the immune system needs to be periodically challenged with the analogues.

Due to genetic variation, different individuals may react with immune responses of varying strength to the same polypeptide. Therefore, the vaccine according to the invention may comprise

several different polypeptides in order to increase the immune response, cf. also the discussion above concerning the choice of foreign T-cell epitope introductions. The vaccine may comprise two or more polypeptides, where all of the polypeptides
5 are as defined above.

The vaccine may consequently comprise 3-20 different analogues, such as 3-10 analogues. However, normally the number of analogues will be sought kept to a minimum such as 1 or 2 analogues.

10 Nucleic acid vaccination

As a very important alternative to classic administration of a peptide-based vaccine, the technology of nucleic acid vaccination (also known as "nucleic acid immunisation", "genetic immunisation", and "gene immunisation") offers a number of at-
15 tractive features.

First, in contrast to the traditional vaccine approach, nucleic acid vaccination does not require resource consuming large-scale production of the immunogenic agent (e.g. in the form of industrial scale fermentation of microorganisms producing proteins). Furthermore, there is no need to devise purification and refolding schemes for the immunogen. And finally, since nucleic acid vaccination relies on the biochemical apparatus of the vaccinated individual in order to produce the expression product of the nucleic acid introduced, the optimum posttranslational processing of the expression product
25 is expected to occur; this is especially important in the case of autovaccination, since, as mentioned above, a significant fraction of the original B-cell epitopes of the polymer should be preserved in the modified molecule, and since B-cell epitopes in principle can be constituted by parts of any
30

(bio)molecule (e.g. carbohydrate, lipid, protein etc.). Therefore, native glycosylation and lipidation patterns of the immunogen may very well be of importance for the overall immunogenicity and this is expected to be ensured by having the host
5 producing the immunogen.

It should be noted that the enhanced expression levels observed with the presently disclosed analogues is very important for efficacy of DNA vaccination, since the in vivo expression level is one of the determining factors in the immunogenic efficacy of a DNA vaccine
10

Hence, a preferred embodiment of the invention comprises effecting presentation of the analogue of the invention to the immune system by introducing nucleic acid(s) encoding the analogue into the animal's cells and thereby obtaining in vivo
15 expression by the cells of the nucleic acid(s) introduced.

In this embodiment, the introduced nucleic acid is preferably DNA which can be in the form of naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formulated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in a polymer, e.g. in PLGA (cf. the microencapsulation technology described in WO 98/31398) or in
20 chitin or chitosan, and DNA formulated with an adjuvant. In this context it is noted that practically all considerations pertaining to the use of adjuvants in traditional vaccine formulation apply for the formulation of DNA vaccines. Hence, all disclosures herein which relate to use of adjuvants in the
25

context of polypeptide based vaccines apply *mutatis mutandis* to their use in nucleic acid vaccination technology.

As for routes of administration and administration schemes of polypeptide based vaccines which have been detailed above, 5 these are also applicable for the nucleic acid vaccines of the invention and all discussions above pertaining to routes of administration and administration schemes for polypeptides apply *mutatis mutandis* to nucleic acids. To this should be added that nucleic acid vaccines can suitably be administered intra- 10 veneously and intraarterially. Furthermore, it is well-known in the art that nucleic acid vaccines can be administered by use of a so-called gene gun, and hence also this and equivalent modes of administration are regarded as part of the present invention. Finally, also the use of a VLN in the administration of nucleic acids has been reported to yield good re- 15 sults, and therefore this particular mode of administration is particularly preferred.

Furthermore, the nucleic acid(s) used as an immunization agent can contain regions encoding the moieties specified in the 20 claims, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at 25 least under the control of different promoters. Thereby it is avoided that the analogue or epitope is produced as a fusion partner to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used, but this is less preferred because of the advantage of ensured co-expression when having 30 both coding regions included in the same molecule.

Accordingly, the invention also relates to a composition for inducing production of antibodies against IL5, the composition comprising

- a nucleic acid fragment or a vector of the invention (cf. the discussion of nucleic acids and vectors below), and
- a pharmaceutically and immunologically acceptable vehicle and/or carrier and/or adjuvant as discussed above.

Under normal circumstances, the nucleic acid is introduced in the form of a vector wherein expression is under control of a viral promoter. For more detailed discussions of vectors and DNA fragments according to the invention, cf. the discussion below. Also, detailed disclosures relating to the formulation and use of nucleic acid vaccines are available, cf. Donnelly JJ et al, 1997, Annu. Rev. Immunol. **15**: 617-648 and Donnelly JJ et al., 1997, Life Sciences **60**: 163-172. Both of these references are incorporated by reference herein.

Live vaccines

A third alternative for effecting presentation of the analogues of the invention to the immune system is the use of live vaccine technology. In live vaccination, presentation to the immune system is effected by administering, to the animal, a non-pathogenic microorganism that has been transformed with a nucleic acid fragment encoding an analogue of the invention or with a vector incorporating such a nucleic acid fragment. The non-pathogenic microorganism can be any suitable attenuated bacterial strain (attenuated by means of passaging or by means of removal of pathogenic expression products by recombinant DNA technology), e.g. *Mycobacterium bovis* BCG., non-pathogenic *Streptococcus* spp., *E. coli*, *Salmonella* spp., Vi-

brion cholerae, *Shigella*, etc. Reviews dealing with preparation of state-of-the-art live vaccines can e.g. be found in Saliou P, 1995, Rev. Prat. 45: 1492-1496 and Walker PD, 1992, Vaccine 10: 977-990, both incorporated by reference herein. For details about the nucleic acid fragments and vectors used in such live vaccines, cf. the discussion below.

As an alternative to bacterial live vaccines, the nucleic acid fragment of the invention discussed below can be incorporated in a non-virulent viral vaccine vector such as a vaccinia strain or any other suitable pox virus.

Normally, the non-pathogenic microorganism or virus is administered only once to the animal, but in certain cases it may be necessary to administer the microorganism more than once in a lifetime in order to maintain protective immunity. It is even contemplated that immunization schemes as those detailed above for polypeptide vaccination will be useful when using live or virus vaccines.

Alternatively, live or virus vaccination is combined with previous or subsequent polypeptide and/or nucleic acid vaccination. For instance, it is possible to effect primary immunization with a live or virus vaccine followed by subsequent booster immunizations using the polypeptide or nucleic acid approach.

The microorganism or virus can be transformed with nucleic acid(s) containing regions encoding the moieties mentioned above, e.g. in the form of the immunomodulating substances described above such as the cytokines discussed as useful adjuvants. A preferred version of this embodiment encompasses having the coding region for the analogue and the coding region for the immunomodulator in different reading frames or at

least under the control of different promoters. Thereby it is avoided that the analogue or epitopes are produced as fusion partners to the immunomodulator. Alternatively, two distinct nucleotide fragments can be used as transforming agents. Of course, having the adjuvating moieties in the same reading frame can provide, as an expression product, an analogue of the invention, and such an embodiment is especially preferred according to the present invention.

Combination treatment

- 10 One especially preferred mode of carrying out the invention involves the use of nucleic acid vaccination as the first (primary) immunization, followed by secondary (booster) immunizations with a polypeptide based vaccine or a live vaccines as described above.

15 Use of the method of the invention in disease treatment

The precise choice of treatment regimen depends on the choice of multimeric protein to target. For instance, when targeting IL5 all conditions discussed in WO 00/65058 are relevant, and when the target is TNF α the diseases/conditions that are relevant are rheumatoid arthritis, juvenile chronic arthritis, spondylarthropathies, polymyositis, dermatomyositis, vasculitis, psoriasis (plaque) and psoriatic arthritis, Mb. Crohn, chronic obstructive pulmonary disorder, myelodysplastic syndrome, uveitis in rheumatoid arthritis, acute pulmonary dysfunction, asthma, Wegener's granulomatosis, irritable bowel disease, temporomandibular disorder (painful jaw joint), stomatitisosteoporosis, and cancer cachexia as well as other inflammatory diseases and other conditions generally appreciated in the art to be linked to the adverse effects of

TNF α . It is therefore possible to treat or ameliorate symptoms that are associated with any of these diseases by employing the method of the invention for down-regulating activity of a multimeric protein.

5 Compositions of the invention

The invention also pertains to compositions useful in exercising the method of the invention. Hence, the invention also relates to an immunogenic composition comprising an immunogenically effective amount of an analogue defined above, said
10 composition further comprising a pharmaceutically and immunologically acceptable diluent and/or vehicle and/or carrier and/or excipient and optionally an adjuvant. In other words, this part of the invention concerns formulations of analogues, essentially as described hereinabove. The choice of adjuvants,
15 carriers, and vehicles is accordingly in line with what has been discussed above when referring to formulation of the analogues for peptide vaccination.

The analogues are prepared according to methods well-known in the art. Longer polypeptides are normally prepared by means of
20 recombinant gene technology including introduction of a nucleic acid sequence encoding the analogue into a suitable vector, transformation of a suitable host cell with the vector, expression of the nucleic acid sequence (by culturing the host cell under appropriate conditions), recovery of the expression
25 product from the host cells or their culture supernatant, and subsequent purification and optional further modification, e.g. refolding or derivatization. Details pertaining to the necessary tools are found below under the heading "Nucleic acid fragments and vectors of the invention" but also in the
30 examples.

Shorter peptides are preferably prepared by means of the well-known techniques of solid- or liquid-phase peptide synthesis. However, recent advances in this technology has rendered possible the production of full-length polypeptides and proteins
5 by these means, and therefore it is also within the scope of the present invention to prepare the long constructs by synthetic means.

Nucleic acid fragments and vectors of the invention

It will be appreciated from the above disclosure that modified
10 polypeptides can be prepared by means of recombinant gene technology but also by means of chemical synthesis or semisynthesis; the latter two options are especially relevant when the modification consists of or comprises coupling to protein carriers (such as KLH, diphtheria toxoid, tetanus toxoid, and
15 BSA) and non-proteinaceous molecules such as carbohydrate polymers and of course also when the modification comprises addition of side chains or side groups to an polymer-derived peptide chain. These embodiments, are, as will be understood from the above, not the preferred ones.

20 For the purpose of recombinant gene technology, and of course also for the purpose of nucleic acid immunization, nucleic acid fragments encoding the analogues are important chemical products (as are their complementary sequences). Hence, an important part of the invention pertains to a nucleic acid frag-
25 ment which encodes an analogue as described herein, i.e. a polymer derived artificial polymer polypeptide as described in detail above. The nucleic acid fragments of the invention are either DNA or RNA fragments.

Most preferred DNA fragment of the invention comprises a nu-
30 cleic acid sequence selected from the group consisting of SEQ

ID NO: 8, 10, 12, 14, 17, 48, 50, 52, 54, 56, and 58 or a nucleic acid sequence complementary to any of these.

The nucleic acid fragments of the invention will normally be inserted in suitable vectors to form cloning or expression
5 vectors carrying the nucleic acid fragments of the invention; such novel vectors are also part of the invention. Details concerning the construction of these vectors of the invention will be discussed in context of transformed cells and microor-
ganisms below. The vectors can, depending on purpose and type
10 of application, be in the form of plasmids, phages, cosmids, mini-chromosomes, or virus, but also naked DNA which is only expressed transiently in certain cells is an important vector (and may be useful in DNA vaccination). Preferred cloning and expression vectors of the invention are capable of autonomous
15 replication, thereby enabling high copy-numbers for the purposes of high-level expression or high-level replication for subsequent cloning.

The general outline of a vector of the invention comprises the following features in the 5'→3' direction and in operable
20 linkage: a promoter for driving expression of the nucleic acid fragment of the invention, optionally a nucleic acid sequence encoding a leader peptide enabling secretion (to the extracellular phase or, where applicable, into the periplasma) of or integration into the membrane of the polypeptide fragment, the
25 nucleic acid fragment of the invention, and optionally a nucleic acid sequence encoding a terminator. When operating with expression vectors in producer strains or cell-lines it is for the purposes of genetic stability of the transformed cell preferred that the vector when introduced into a host cell is in-
30 tegrated in the host cell genome. In contrast, when working with vectors to be used for effecting *in vivo* expression in an

animal (i.e. when using the vector in DNA vaccination) it is for security reasons preferred that the vector is not incapable of being integrated in the host cell genome; typically, naked DNA or non-integrating viral vectors are used, the
5 choices of which are well-known to the person skilled in the art.

The vectors of the invention are used to transform host cells to produce the modified IL5 polypeptide of the invention. Such transformed cells, which are also part of the invention, can
10 be cultured cells or cell lines used for propagation of the nucleic acid fragments and vectors of the invention, or used for recombinant production of the modified IL5 polypeptides of the invention. Alternatively, the transformed cells can be suitable live vaccine strains wherein the nucleic acid frag-
15 ment (one single or multiple copies) have been inserted so as to effect secretion or integration into the bacterial membrane or cell-wall of the modified IL5.

Preferred transformed cells of the invention are microorganisms such as bacteria (such as the species *Escherichia* [e.g.
20 *E. coli*], *Bacillus* [e.g. *Bacillus subtilis*], *Salmonella*, or *Mycobacterium* [preferably non-pathogenic, e.g. *M. bovis* BCG]), yeasts (such as *Saccharomyces cerevisiae*), and protozoans. Alternatively, the transformed cells are derived from a multicellular organism such as a fungus, an insect cell, a plant
25 cell, or a mammalian cell. Most preferred are cells derived from a human being, cf. the discussion of cell lines and vectors below. Recent results have shown great promise in the use of a commercially available *Drosophila melanogaster* cell line (the Schneider 2 (S₂) cell line and vector system available
30 from Invitrogen) for the recombinant production of IL5 analogues of the invention, and therefore this expression system

is particularly preferred, and therefore this type of system is also a preferred embodiment of the invention in general.

For the purposes of cloning and/or optimized expression it is preferred that the transformed cell is capable of replicating the nucleic acid fragment of the invention. Cells expressing
5 the nucleic acid fragment are preferred useful embodiments of the invention; they can be used for small-scale or large-scale preparation of the analogue or, in the case of non-pathogenic bacteria, as vaccine constituents in a live vaccine.

10 When producing the analogues of the invention by means of transformed cells, it is convenient, although far from essential, that the expression product is either exported out into the culture medium or carried on the surface of the transformed cell, since both of these options facilitate subsequent
15 purification of the expression product.

When an effective producer cell has been identified it is preferred, on the basis thereof, to establish a stable cell line which carries the vector of the invention and which expresses the nucleic acid fragment encoding the modified IL5. Preferably,
20 ly, this stable cell line secretes or carries the IL5 analogue of the invention, thereby facilitating purification thereof.

In general, plasmid vectors containing replicon and control sequences that are derived from species compatible with the host cell are used in connection with the hosts. The vector
25 ordinarily carries a replication site, as well as marking sequences which are capable of providing phenotypic selection in transformed cells. For example, *E. coli* is typically transformed using pBR322, a plasmid derived from an *E. coli* species (see, e.g., Bolivar et al., 1977). The pBR322 plasmid contains
30 genes for ampicillin and tetracycline resistance and thus pro-

vides easy means for identifying transformed cells. The pBR plasmid, or other microbial plasmid or phage must also contain, or be modified to contain, promoters that can be used by the prokaryotic microorganism for expression.

5 Those promoters most commonly used in prokaryotic recombinant DNA construction include the B-lactamase (penicillinase) and lactose promoter systems (Chang et al., 1978; Itakura et al., 1977; Goeddel et al., 1979) and a tryptophan (trp) promoter system (Goeddel et al., 1979; EP-A-0 036 776). While these are
10 the most commonly used, other microbial promoters have been discovered and utilized, and details concerning their nucleotide sequences have been published, enabling a skilled worker to ligate them functionally with plasmid vectors (Siebwenlist et al., 1980). Certain genes from prokaryotes may be expressed
15 efficiently in *E. coli* from their own promoter sequences, precluding the need for addition of another promoter by artificial means.

In addition to prokaryotes, eukaryotic microbes, such as yeast cultures may also be used, and here the promoter should be capable of driving expression. *Saccharomyces cerevisiae*, or
20 common baker's yeast is the most commonly used among eukaryotic microorganisms, although a number of other strains are commonly available. For expression in *Saccharomyces*, the plasmid YRp7, for example, is commonly used (Stinchcomb et
25 al., 1979; Kingsman et al., 1979; Tschemper et al., 1980). This plasmid already contains the *trp1* gene which provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan for example ATCC No. 44076 or PEP4-1 (Jones, 1977). The presence of the *trp1* lesion as a
30 characteristic of the yeast host cell genome then provides an

effective environment for detecting transformation by growth in the absence of tryptophan.

Suitable promoting sequences in yeast vectors include the promoters for 3-phosphoglycerate kinase (Hitzman et al., 1980) or
5 other glycolytic enzymes (Hess et al., 1968; Holland et al., 1978), such as enolase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose
10 cose isomerase, and glucokinase. In constructing suitable expression plasmids, the termination sequences associated with these genes are also ligated into the expression vector 3' of the sequence desired to be expressed to provide polyadenylation of the mRNA and termination.

15 Other promoters, which have the additional advantage of transcription controlled by growth conditions are the promoter region for alcohol dehydrogenase 2, isocytochrome C, acid phosphatase, degradative enzymes associated with nitrogen metabolism, and the aforementioned glyceraldehyde-3-phosphate dehydrogenase, and enzymes responsible for maltose and galactose
20 utilization. Any plasmid vector containing a yeast-compatible promoter, origin of replication and termination sequences is suitable.

In addition to microorganisms, cultures of cells derived from
25 multicellular organisms may also be used as hosts. In principle, any such cell culture is workable, whether from vertebrate or invertebrate culture. However, interest has been greatest in vertebrate cells, and propagation of vertebrate in culture (tissue culture) has become a routine procedure in recent
30 years (Tissue Culture, 1973). Examples of such useful

host cell lines are VERO and HeLa cells, Chinese hamster ovary (CHO) cell lines, and W138, BHK, COS-7 293, *Spodoptera frugiperda* (SF) cells (commercially available as complete expression systems from i.a. Protein Sciences, 1000 Research
5 Parkway, Meriden, CT 06450, U.S.A. and from Invitrogen), and MDCK cell lines. In the present invention, an especially preferred cell line the insect cell line S₂, available from Invitrogen, PO Box 2312, 9704 CH Groningen, The Netherlands.

Expression vectors for such cells ordinarily include (if necessary) an origin of replication, a promoter located in front
10 of the gene to be expressed, along with any necessary ribosome binding sites, RNA splice sites, polyadenylation site, and transcriptional terminator sequences.

For use in mammalian cells, the control functions on the expression vectors are often provided by viral material. For example, commonly used promoters are derived from polyoma, Adenovirus 2, and most frequently Simian Virus 40 (SV40) or cytomegalovirus (CMV). The early and late promoters of SV40 virus are particularly useful because both are obtained easily
20 from the virus as a fragment which also contains the SV40 viral origin of replication (Fiers et al., 1978). Smaller or larger SV40 fragments may also be used, provided there is included the approximately 250 bp sequence extending from the *HindIII* site toward the *BglI* site located in the viral origin
25 of replication. Further, it is also possible, and often desirable, to utilize promoter or control sequences normally associated with the desired gene sequence, provided such control sequences are compatible with the host cell systems.

An origin of replication may be provided either by construction of the vector to include an exogenous origin, such as may
30

be derived from SV40 or other viral (e.g., Polyoma, Adeno, VSV, BPV) or may be provided by the host cell chromosomal replication mechanism. If the vector is integrated into the host cell chromosome, the latter is often sufficient.

5 EXAMPLE 1

Design of 4 new two-epitope (P2+P30) monomer IL5 constructs

IL5 is an anti-parallel homo-dimer, in which the C termini and N termini of the monomers are located closely in the molecule. This opens for the possibility of linking the two monomers into a single monomer, closely resembling the wild-type dimer quarternary structure.

We have approached this using either the p2/P30 epitopes as linker or by inserting a di-glycine linker as described previously in Li et al. 1997, PNAS USA 94(13): 6694-9.

15 The native hIL5 encoding DNA molecule used in all the research work was purchased from R&D systems (BBG16). This DNA sequence did not include the hIL5 leader sequence; hence was added a synthetic DNA sequence encoding the natural hIL5 leader peptide. The sequences encoding the P2 and P30 T cell epitopes
20 are derived from tetanus toxoid. These sequences were inserted into the native sequence of the gene thus providing the immunogenic variants of IL5. The insertions are made preserving the reading frame in the IL5 gene.

The cloning strategy for making the variants is based on elongation of primers or DNA fragments with sequence overlap.

First, two sets of primers with complementary 5' ends making up the insertion are elongated in two separate PCR reactions

using the wt IL5 DNA molecule as template and a flanking vector primer. Thereafter, these two double stranded fragments, which accordingly also have complementary 5' ends, are annealed and elongated to include the complete insert in a second PCR. Finally, the fragment is amplified using the flanking primers. These inserts are then digested with the appropriate endonucleases, as is the vector and vector and inserts are ligated together. This procedure is a modification of the "splice by overlap extension" procedure described by Horton et al. 1989 and outlined in Current protocols in molecular biology (pp. 8.5.7-9) "Introduction of a point mutation by sequential PCR steps" by Ausabel et al.

Standard molecular biological techniques and DNA manipulations such as restriction enzyme digests, agarose gel electrophoresis, growth and storage of the *E. coli* cells were performed using standard molecular biological techniques described in the laboratory manual Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989 and using the M&E standard protocols

EXAMPLE 2

20 *hIL5.34 and hIL5.35*

In order to have the T-cell epitopes internally in the molecule, P2 and P30 are inserted head to tail as a linker between the two IL5 monomers thereby giving rise to two constructs *hIL5-P30-P2-hIL5* (*hIL5.34*, mature peptide in SEQ ID NOs: 5 and 6) and *hIL5-P2-P30-hIL5* (*hIL5.35*, mature peptide in SEQ ID NOs: 7 and 8) - both DNA constructs encode the natural IL5 leader sequence, resulting in a mature expression product of 266 amino acids. The translation products resulting from these designs are intended to fold into a "monomeric IL5 dimer",

i.e. a monomeric molecule that has a tertiary structure that very much resembles the complete 3-dimensional structure of dimeric IL5.

EXAMPLE 3

5 hIL5.36 & hIL5.37

Based on the previous successful generation of a biologically active monomer "IL5 dimer mimic" by insertion of a di-glycine-linker by J. Li et al., similar, but immunogenic, construct with the addition of T-cell epitopes were designed. The variant hIL5.36 thus has the structure of the mature peptide in SEQ ID NOs: 9 and 10 and variant hIL5.37 has the structure of the mature peptide in SEQ ID NOs: 11 and 12. Both these constructs encode a natural IL5 leader sequence followed by the first 4 amino acids in IL5 that in turn is followed by the first inserted epitope - the other epitope is positioned in the C-terminus.

There are 2 main reasons that the N-terminal epitope is not positioned N-terminally to the complete IL5 sequence in these two constructs instead of aiming at preserving the hIL5 sequence. By using the natural hIL5 leader peptide together with the N-terminus of hIL5 we ensure that the leader peptide is cleaved off correctly. And, since the N-terminus in IL5 constitutes a flexible region, it is not of significance for preservation of 3-dimensional structure of the resulting construct.

The translation products resulting from these designs are seen to fold into a "monomeric IL5 dimer" as described in Example 2.

EXAMPLE 4

Expression levels

The above described human IL5 analogues have been inserted into multiple vectors, used for construction, DNA vaccination
5 and, recombinant expression in insect-, mammalian- or *E. coli* cells using standard methods in the art.

Especially, using standard expression systems and protocols in COS cells (transient expression) and in S₂ cells, it was found that the expression levels were even better than those obtained
10 ned with constructs encoding IL5 wildtype protein and the expression levels also exceeded those obtained when expressing the hIL5 variants disclosed in WO 00/65058.

EXAMPLE 5

Induction of anti-IL5 cross-reactive antibodies

15 The presently disclosed analogues of human IL5 where used in standard immunization protocols of mice. In brief, mice were immunized with the above-described variants from examples 2 and 3. The murine anti hIL5 antibodies were isolated via immunoaffinity chromatography and their anti-hIL5 activity was
20 compared to that of murine antibodies raised against wild-type hIL5. The results indicated that the antibodies were higher titered and also of higher affinity than antibodies against the analogues taught in WO 00/65058.

Preliminary results also indicate that the multimer mimics according to the present invention have preserved at least some
25 of the IL5 specific activity.

EXAMPLE 6

Preparation of TNF α variants

A synthetic DNA sequence "SMTNFWT3" (SEQ ID NO: 16) encoding the wild type human TNF α monomer polypeptide (SEQ ID NO: 17) was delivered as a ligation product from Entelechon GmbH. The DNA sequence of the human hTNF α was optimised for expression in *E. coli* according to the Codon Usage Database by exclusion of all codons with a frequency in *E. coli* of less than 10%. Further, the sequence was designed to include a 5' NcoI restriction site for subsequent cloning steps.

The SMTNFWT3 ligation product was introduced into the pCR 4 TOPO Blunt vector and *E. coli* DH10B cells were transformed. Plasmid DNA from 10 of the resulting SMTNFWT3TOPO clones was purified and five clones containing the expected fragment (when analysed by Restriction Enzyme (RE) digest) were selected.

The NcoI/EcoRI DNA fragments from the five potentially correct SMTNFWT3TOPO clones were isolated and transferred to the pET28b(+) vector and sequence determined. Insertions, deletions or substitutions were identified in four clones whereas one clone appeared to be correct. The correct construct - SMTNFWT3pET28 was subsequently used as template for the generation of all single TNF α variants.

EXAMPLE 7

TNF34 construction

The PanDR epitope amino acid sequence (SEQ ID NOs: 7 and 20) was manually "reverse-translated" to a DNA sequence (SEQ ID NO: 19) optimised for expression in *E. coli*, see below, and inserted in loop 3 of TNF α by SOE PCR.

The resulting construct (a DNA sequence encoding SEQ ID NO: 18) was placed in the pET28b+ vector to generate TNF34-pET28b+.

10 EXAMPLE 8

Monomerized trimer construction

The monomerized trimer constructs are based on 3 TNF α encoding regions, separated by either a tri-glycine linker and/or an epitope encoding region.

- 15 The TNF- α gene was synthesized as three separate entities. The three fragments were assembled by SOE PCR, and the assembled gene (SEQ ID NO: 21) was cloned into pCR2.1-TOPO. After sequence verification, a correct clone was isolated. The hTNFT_0 gene (SEQ ID NO: 21 encoding TNF α -GlyGlyGly-TNF α -GlyGlyGly-
20 TNF α , SEQ ID NO: 22, i.e. 3 copies of SEQ ID NO: 17 separated by two tri-glycine linkers) was then transferred to pET28b+ to generate hTNFT_0-pET28b+. A correct clone was isolated, sequence verified and transformed into *E. coli* lines BL21-STAR, BL21-GOLD and HMS174.

hTNFT_0-pET28b+ was used as template to generate the following four monomerized trimer variants: hTNFT_1, hTNFT_2, hTNFT_3 and hTNFT_4 (SEQ ID NOs: 49, 51, 57, and 59) by SOE PCR. A further variant (SEQ ID NO: 53) can be made in a similar way.

5 hTNFT_1, hTNFT_2 and hTNFT_3 are variants including tetanus toxoid epitopes P2 and P30 (SEQ ID NOs: 3 and 5, respectively) that need to be assembled by two rounds of SOE PCR. hTNFT_4 is a variant with a PADRE (SEQ ID NO: 7) insert and can be assembled by a single round of SOE PCR. A further variant (SEQ ID
10 NO: 55) can be made in a similar way.

hTNFT_4 was constructed by the above mentioned methods, and a correct clone of hTNFT_4-pET28b+ was found in TOP 10 cells and the construct was transferred to BL21-STAR and HMS174 cells.

To generate hTNFT_1, hTNFT_2 and hTNFT_3 the epitopes were in-
15 serted by SOE PCR in very small fragments of the trimer, which were inserted into hTNFT_0-pET28b+ by RE cutting and ligation.

EXAMPLE 9

Stabilising TNF34 mutants

To further stabilise the TNF34-pET28b+ variant described
20 above, variants containing the introduction of an extra disulfide bridge as well as a deletion mutant were constructed. 3 different variants were constructed:

TNF34-A-pET28b+ contains the substitutions Q67C and A111C,
TNF34-B-pET28b+ contains A96C and I118C, and TNF34-C-pET28b+
25 that contains a deletion of the 8 most N-terminal amino acids

- the amino acid sequences of the expression products are set forth in SEQ ID NOs: 20, 30, and 31.

All 3 constructs were made using SOE PCR, and were cloned in BL21-STAR, BL21-GOLD and HMS174, followed by sequence verification.
5 cation.

EXAMPLE 10

Flexible loop variants

In order to find a variant that might exhibit improved characteristics compared to the TNF34-pET28b+ variant, constructs
10 were made where the PADRE insert (SEQ ID NO: 7) is moved around in flexible loop 3 of the TNF- α molecule.

All of these: TNF35-pET28b+, TNF36-pET28b+, TNF37-pET28b+, TNF38-pET28b+, TNF39-pET28b+, and a variant with PADRE placed in the C terminus of the molecule; TNFC2-pET28b+, were made
15 with SOE PCR technique and were cloned in BL21-STAR, BL21-GOLD and HMS174, followed by sequence verification. The amino acid sequences of the expression products are set forth in SEQ ID NOs: 23, 24, 24, 26, 27 and 28.

To also evaluate the possibility of using insect cells as expression system, TNFWT, TNF34, TNF35, TNF36, TNF37, TNF38, TNF39 and TNFC2 were transferred into the p2Zop2f vector (cf. Fig. 1), and expressed in S2 insect cells.
20

EXAMPLE 11

Other constructs

A large number of further TNF α variants have been prepared, all termed TNFX, cf. above. The DNA encoding these variants
5 has being made by SOE PCR, and cloned directly into pET28b+.

The correct TNFX clones have been transformed into BL21-STAR and HMS174, and subsequently sequence verified.

EXAMPLE 12

Periplasmic expression

- 10 The LTB leader sequence has been added directly upstream of SEQ ID NO: 16 in TNF34-pET28b+, to target the expression to the periplasmic space.

EXAMPLE 13

Mammalian expression

- 15 To test for expression in mammalian cells, SEQ ID NO: 16 and the DNA encoding TNF34 have been transferred to the pHP1 vector, which is a variant of the commercially available pCI vector (Promega Corporation). pHP1 includes a kanamycin resistance gene as marker instead of the AmpR gene of pCI.

EXAMPLE 14

Co-expression of GroEL and GroES

The expression of the *E. coli* chaperone complex, GroEL/ES, has previously been reported to increase the expression of soluble
5 TNF α mutants (Jeong, W et al 1997, Biotechnology letters, vol 19, no 6 pp579-582). To test if the coexpression of GroEL/ES could improve the expression of the TNF α variants as herein described, a plasmid containing the GroEL/ES operon from *E. coli* under control of its natural promoter has been used. This
10 plasmid has been co-transformed into HMS174 together with either DNA encoding wtTNF α , TNF34 or TNF37. Double transformants were selected by plating out on plates containing both Kanamycin and Carbecillin, which are the two relevant selection markers. Double transformants were then identified by RE
15 analysis to test for the presence of both plasmids in the same clone.

In a pilot experiment, cells were grown at 37°C to OD600= 0.6 -1 followed by a 30 min heatshock at 42°C. A control fraction of the cells were not heatshocked, and all cells were diluted
20 5 times into LB media containing 1 mM IPTG and grown ON at 25°C.

The cells were harvested and both supernatants and lysates were analysed for TNF α expression. Commassie staining was performed to evaluate the GroEL/ES expression.

25 In this experiment, no improvement by addition of chaperones was observed. This is mainly because we obtain almost 100% soluble material in this experiment, event in the absence of chaperones. We will however check the improvement on other

variants of the invention if these appear to be less soluble variants.

EXAMPLE 15

E. coli expression

5 Expression of soluble TNF α variants in three different *E. coli* strains has been tested in laboratory fermentors as well as in shake flasks. The fermentation equipment used was the Infors fermentor system with 1L working volume. The three *E. coli* strains tested were: HMS174, BL21 STAR and BL21 GOLD. The me-
10 dium used for the fermentations was a defined minimal medium with glucose as the sole carbon source.

One of the primary objectives was to determine optimum fermentation process parameters (especially temperature and IPTG concentration) so as to optimise for expression of soluble
15 TNF α variants.

Process Parameters:

Parameter	Set point	Range	Action limit
pH	7.0	6.5 - 7.5	< 6.4 - > 7.6
Temp. start	37 °C	36 - 38 °C	< 36 - > 38 °C
Temp. induction	25 °C	24 - 26 °C	< 24 - > 26 °C
DO ₂ tension	30 %	0 - 100 %	> 90 % for more than 4 hours
Stirrer	1000 RPM	1000 - 1500	-

It has been found that one suitable scheme is the following:
The IPTG concentration is 0,5 mM and the temperature at induc-
20 tion is lowered to 25 °C. The total fermentation time is between 14 and 18 hours, including propagation, induction and

protein production. The total fermentation time depends on the growth of the culture. OD600 start in the fermentor is typically between 0,1 - 0,3 (2-6 in the pre culture) as calculated from the OD in the inoculation culture. Induction of culture
5 is performed at OD600 = 20 ± 1-2 or nine to eleven hours after inoculation. Protein production then takes place for three to five hours.

Alternatively: Expression of TNF- α variant is accomplished by taking advantage of a low temperature culture to avoid intra-
10 cellular precipitation of the variant protein to inclusion bodies. Growth of the culture to the wanted OD is done at the same temperature as the actual induction to avoid "shocks" to the cells by changing the temperature from the optimal growth temperature (37°C) to the lower induction temperature (25°C).
15 By using this method it is believed that the only pressure imposed on the cells is the actual induction by IPTG - at any rate, this method has recently provided significantly improved yields of soluble expression product. By making a small overnight culture and preparing the larger 1L LB medias a day in
20 advance the generation of material in the large LB-cultures can be accomplished in approximately 9 hours while the actual induction period is done over night (in 16-20 hours). Hence, a preferred method can be described as follows: Expression of the TNF α variant is performed in 2x2 L baffled shake flasks
25 containing 1 L LB media, each with the only modification to the above-mentioned method being that cells (BL21 STAR) are grown at 25°C to an OD₄₃₆ of 0.7 after which the cells are induced with 1 mM IPTG and allowed to produce protein for 20 hours (still at 25°C).

EXAMPLE 16

Selection assays

A direct receptor ELISA together with a polyclonal ELISA and a cytotoxicity assay with KD-4 and Wehi cells are used as first
5 line assays to screen and follow purification. Antibodies produced against TNF α variants are used to inhibit wtTNF α binding in both the receptor and the cytotoxic assay, to measure the antibody quality.

EXAMPLE 17

10 *Purification Procedures*

In this example, recombinant production and subsequent purification of one of the TNF α variants (TNF37) is described in detail. However, the purification procedure is the preferred one according to the present invention and will also be
15 applicable (with small adjustments relevant for each variant) for other TNF α variants of the present invention.

An *E. coli* strain BL21 STAR/TNF37 colony from a LB-kanamycin plate (60 mg kanamycin/L LB media containing 1.5 % Agar) is resuspended in 5 ml LB-media (60 mg kanamycin/L LB) and grown
20 over night (16 hours) at 37°C while shaking 220 RPM in a New Brunswick shaker.

2x2 ml of this culture is transferred to 2x1 L LB (60 mg kanamycin/L) in 2L baffled shake flasks and the cells are allowed to grow in a New Brunswick shaker at 220 RPM to OD₄₃₆ = 0.6-
25 0.8. This step has been performed at the exemplary

temperatures 37°C and 25°C, but the temperature may be optimised for each culture.

1 ml 1 M IPTG is added to each flask and the cells are allowed to grow for 16-20 hours. Before induction, the temperature is
5 adjusted to 25°C if this is not already the fermentation temperature.

The cells are harvested in centrifuge tubes (500 ml) by centrifugation at 5000 RPM for 15 min using an SLA-3000 head in a Sorvall centrifuge.

10 The cells are transferred to one 500 ml pre-weight centrifuge tube using 0.9 % NaCl and harvest cells by centrifugation as before.

The supernatant is discharged and the tube is weighed to determine the cell weight (should be 7-11 grams).

15 200 ml 50 mM Na₂HPO₄, pH = 7.0 is added (if cells are re-suspended they should be used directly, otherwise it is possible to freeze).

Cell disruption, centrifugation, and filtration

A mechanical disruption of the cells offer several advantages
20 over enzymatic disruption in terms of efficiency, reliability and the ability to choose any buffer necessary in the following steps of the purification. The APV-1000 is kept cool during the operation by adding ice water to the sample-chamber before use and pas ice water through the machine between the
25 two passages of sample. Centrifugation and filtration serves to remove any particles or aggregates from solution prior to chromatographic separation of the proteins. The cell disruption

tion and HA-chromatography should be done the same day as this might minimize the apparent protease activity as a consequence of the separation from these in the chromatographic step. The procedure for disruption, centrifugation and filtration is as follows:

The carefully re-suspended cell material is transferred from to the cell-disrupter (APV-1000). The cell-suspension is carefully passed 2x through the disrupter (cooling on ice after each passage and passing ice water through the APV-1000 in between the passages) using 700 bars of backpressure (the solution ought to be clear at this point).

The disrupted cells are transferred to a 500 ml centrifuge tube and the cells are spun for 45 min at 10000 RPM in a Sorvall centrifuge using the SLA-3000 head.

15 The extract (approx 225 ml) is passed through a 0.22 μ m filter.

Hydroxyapatite(HA) chromatography

Hydroxyapatite Bio-Gel HTP Gel (BIO-RAD; catalog # 130-0420) is a crystalline form of calcium phosphate having proven itself as a unique tool in the separation of proteins such as monoclonal antibodies and other proteins otherwise not separable by other methods. However, in our experience the flow properties of the material are somewhat critical in that sense that a flow higher than 2 ml/min raises the pressure to an unacceptable high level. Also the material has collapsed several times when attempt has been made to regenerate with sodium hydroxide as recommended by the manufacturer.

Buffers and Column

Stock for buffer A + B: 1 M $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, pH = 7.0 (pH ad-

justed to 7 with HCl). Buffers A+B made from dilutions of stock.

Buffer A: 50 mM $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, pH = 7.0

Buffer B: 0.3 M $\text{Na}_2\text{HPO}_4 \times 2\text{H}_2\text{O}$, pH = 7.0

- 5 Column packed to approximately 50-60 ml with hydroxyapatite Bio-Gel HTP Gel (BIO-RAD; catalog # 130-0420) using a suspension in Buffer A and a XK 26/40 (Amersham Biosciences) column.

Chromatography Program

- 10 Purge system 20 ml at a flow of 30 ml/min.
Equilibration: 4 CV of Buffer A at a flow of 2 ml/min
Load sample through pump (inlet F on the BioCad) (approx 225+5-10 ml if the sample in the tubing is needed) at a flow of 2 ml/min.
- 15 Wash column with 1.5 CV Buffer A at a flow of 2 ml/min.
Elution: Elute protein with a gradient of 4 CV from 0 % to 100 % Buffer B at a flow of 2 ml/min.
Clean column with 2 CV Buffer B at a flow of 2 ml/min.
Re-equilibration with 4 CV Buffer A at a flow of 2 ml/min.
- 20 Select fractions, pool, and dialyse ON at 4 °C against 15x volume 20 mM Tris-HCl, 0.075 M NaCl, pH 8.0.

Selecting TNF37-containing Fractions after HA chromatography

- The HA chromatography elution fraction profile basically consist of a "run through" fraction and one eluted peak that can
- 25 be separated into several peaks. The TNF37-containing fractions has to be selected on the basis of a coomassie stained gel of the entire peak since a peak containing TNF37 is not directly identifiable. However, as a consequence of subsequent purification steps the selection of fractions at this point is
- 30 less critical and it is possible to remove contaminants later

in the procedure. Thus, a less conservative selection of fractions ensures maximum yield of variant.

Initially the "run through" was checked with "dot blots" for any TNF37. This gave a positive result that in theory should indicate that a significant part of the variant did not bind to the column. However, when the "run through" is subjected to the very efficient SP-sepharose Cation Exchange Chromatography (cf. next step) and the fractions are analysed with coomassie stained gels they do not contain any detectable TNF37-variant indicating some false positive reaction in the "dot blot" or a fraction of the variant that binds completely different to the SP-sepharose.

SP-sepharose Cation Exchange Chromatography

SP-sepharose is a basic cation exchange step selected as consequence of the rather high calculated pI of 9.4 of the variant compared to the wtTNF α pI of 7.8. This increase in pI is a consequence of the 2 lysines introduced via the PADRE epitope. This chromatography is very efficient and fast for the TNF37 variant and is expected to be useful for a large number of other loop variants of TNF α .

The sample applied should have a lower conductivity than 8 mS/cm and pH should be at least 7.7 before continuing with SP-sepharose chromatography since variations from this in our experience has made the binding properties of the protein different from time to time.

Buffers and Column

Stocks to buffers A+B: 1 M Tris-HCl. pH = 8.0.

Buffer A: 20 mM Tris-HCl, 0.075 M NaCl, pH = 8.0.

Buffer B: 20 mM Tris-HCl, 1 M NaCl, pH = 8.0.

Column packed to approximately 60 ml with SP-sepharose FF (Amersham Biosciences; catalogue # 17-0729-01) using a suspension in Buffer A and a XK 26/40 (Amersham Biosciences) column.

5 Chromatography Program

Purge system 20 ml at a flow of 30 ml/min

Equilibration: 4 CV of Buffer A at a flow of 4 ml/min.

Load sample through pump (inlet F on the BioCad) (Sample+10 ml if the sample in the tubing is needed) at a flow of 4 ml/min.

Wash column with 1.5 CV Buffer A at a flow of 4 ml/min.

Elution: Elute protein with a gradient of 4 CV from 0 % to 100 % Buffer B at a flow of 4 ml/min.

Clean column with 2 CV Buffer B at a flow of 4 ml/min.

15 Re-equilibration with 4 CV Buffer A at a flow of 4 ml/min.

Select fractions, pool, and dialyse ON at 4 °C against 15x volume 20 mM Tris-HCl, 0.075 M NaCl, pH 8.0.

Selecting TNF37 containing Fractions after SP Sepharose chromatography

20 The profile basically consists of a "run through" fraction and several protein containing peaks. However two peaks contains the variant with some contaminants. It is at this point important not to include too many fractions on the right side of peak two since this in our experience includes too many contaminants that are not easily removed in subsequent chromatographic steps.

Q-sepharose Anion Exchange Chromatography

Q-sepharose is a basic anion exchange step selected for removing a major contaminant protein that with high reproducibility

lity follows the purification of TNF37 including the HA-chromatography and SP-sepharose. The TNF37 variant itself does not bind to the column but the major unknown contaminant does. It is, however, possible to select fractions in a conservative
5 fashion already in the SP-sepharose step in that way avoiding the contaminant. However, this compromises the yield of TNF37 variant compared to when the Q-sepharose is used in the procedure and since also other minor contaminants are removed in this step, it is preferred to include it in the total procedure.
10 In conclusion the Q-sepharose step is important in the purification of variant 37 and offers an even better end product with a high yield.

Buffers and Column

Stocks to buffers A+B: 1 M Tris-HCl. pH = 8.0.
15 Buffer A: 20 mM Tris-HCl, 0.075 M NaCl, pH = 8.0.
Buffer B: 20 mM Tris-HCl, 1 M NaCl, pH = 8.0.
Column packed to approximately 50-60 ml with Q-sepharose FF (Amersham Biosciences; catalogue # 17-0510-01) using a suspension in Buffer A and a XK 26/40 (Amersham Biosciences)
20 column.

Chromatography program

Purge system 20 ml at a flow of 30 ml/min.
Equilibration: 4 CV of Buffer A at a flow of 4 ml/min
Load sample through pump (inlet F on the BioCad) (Sample+10
25 ml if the sample in the tubing is needed) at a flow of 2 ml/min.
Wash column with 3 CV Buffer A at a flow of 4 ml/min.
Elution: Elute remaining protein with 2 CV 100 % Buffer B at a flow of 4 ml/min.
30 Re-equilibration with 4 CV Buffer A at a flow of 4 ml/min.

Select fractions, pool and apply directly on SP-sepharose column.

The elution profile basically consists of a "Run through" fraction and several protein containing peaks. The "Run through" fraction can sometimes be divided into several purely resolved peaks which all contains the TNF37 variant and therefore all are pooled. This heterogeneity of the TNF37 is probably solved when the problem with the apparent proteolytic degradation is solved.

10 EXAMPLE 18

Immunisation studies

Materials:

- Saline (0,9% NaCl in sterile water, Fresenius Kabi Norge AS, Norway)
- 15 Complete Freund's Adjuvant (Sigma, F-5881, 39H8926)
Incomplete Freund's Adjuvant (Sigma, F-5506, 60K8937)
Alhydrogel 2% [10 mg Al/ml] (Brenntag Biosector, Batch 96 (3176))
Adjuphos [5 mg Al/ml] (Brenntag Biosector, Batch 2 (8937))
- 20 Wild type human TNF (Invitrogen cat.no:10062-024).
KYM-1D4: Provided by A. Meager (A. Meager, J. Immunol. Methods 1991, 144:141-143)
WEHI 164 clone 13: Provided by T. Espevik (T. Espevik and J. Nissen-Myer, J. Immunol. Methods 1986, 95:99-105)
- 25 Tetrazolium salt (MTS, CellTiter 96 Aqueous one solution; Promega, G3581)
Rotating bar (Rotamix, Heto, Denmark)
Vortex (OLE DICH instrumentmakers ApS, Denmark)

Choice of formulation / adjuvant

The purified TNF α variant proteins (in 20 mM Tris-HCl, 0.075 M NaCl, pH 8.0) are diluted to 0,5 mg/ml with saline (0,9% NaCl), batched (375 μ g/vial) and stored at -20°C until used
5 for immunizations.

For each TNF variant, immunizations are made with two adjuvants: 1) Complete Freund's Adjuvant (CFA, for the primary immunization) and Incomplete Freund's Adjuvant (IFA, for boost immunizations) and 2) Alhydrogel or Adjuphos (state-of-the-art
10 Aluminium hydroxide and aluminium phosphate adjuvants, respectively) - these are used for both prime and boost injections.

Before primary immunization, a decision on the choice of either Alhydrogel or Adjuphos as adjuvant for the TNF variant is made. The adjuvant with the best ability to adsorb the TNF
15 variant is chosen for further use in the immunization experiment. Two aliquots of the TNF α variant are mixed with an equal volume of Alhydrogel and Adjuphos in two vials. The vials are gently mixed at room temperature for 30 minutes on a rotating bar. Vials are then centrifuged at 13000 g for 15 minutes and
20 supernatant is tested for the soluble TNF variant content on a gradient (4-12%) SDS gel. The adjuvant/variant aliquot containing the least free variant (i.e. where more variant has bound to aluminium-particles) is then selected as the best adjuvant.

25 Preparation of antigen/adjuvant emulgate:

CFA/IFA emulgates are prepared through the following procedure:

Vials with TNF α variant [0,5 mg/ml] is thawed, transferred to a 10 ml sterile vial and mixed with an equal volume of CFA or IFA. The vial is then mixed further on a vortex at 3300 rpm for 30 minutes at 20°C.

- 5 Alhydrogel/Adjuphos emulgates are prepared through the following procedure:

Alhydrogel/Adjuphos are diluted to 1,4 mg Al/ml with saline. Vials with TNF α variant [0,5 mg/ml] is thawed, transferred to a 10 ml sterile vial and mixed with an equal volume of Alhydrogel [1,4 mg Al/ml] or Adjuphos [1,4 mg Al/ml]. The vial is then mixed further on a rotating bar for 30 minutes at 20°C.

Choice of animal model

Six - eight weeks old Balb/Ca female mice are repetitively immunized with TNF α variants. Blood samples are collected at 15 different intervals and isolated sera are investigated for anti-wtTNF α antibody titers. Mice are ordered from Taconic Farms, Inc. Acquires M&B A/S, Denmark. Mice are housed at the animal facility of Pharmexa for one week before initiation of experiment.

20 Immunization scheme and dosage

Groups of 10 + 10 mice are immunized with each TNF α variant in CFA/IFA and Alhydrogel/Adjuphos respectively. 20 + 20 mice are used for immunization with wild type TNF α .

At the first immunization, 50 μ g of protein in adjuvant will 25 be injected subcutaneously. All mice will receive additional

booster immunizations subcutaneously with 25 µg of protein in adjuvant 2, 6 and 10 weeks after the first immunization.

Blood samples will be collected immediately before the first immunization and 1 week after each boost immunization.

5 Assays employed

Cytotoxicity bioassay using WEHI 164 clone 13- or KYM-1D4-cells: This assay is used to determine the toxicity of TNFα variants of the invention. Cells are cultured for 48 hours in the presence of titrated amounts of TNFα variants and cell death is determined by addition of Tetrazolium salt (MTS), which is bio-reduced into a coloured formazan product by living cells. Cytotoxicity of TNFα variants are compared to that of human wild type TNFα.

Cytotoxicity-inhibition bioassay using WEHI 164 clone 13- or KYM-1D4-cells: This assay is used to investigate the ability of anti-sera raised in TNFα immunized mice to neutralize the cytotoxic effect of wild type TNFα. Cells are cultured for 48 hours with titrated amounts of anti-sera and a constant concentration of wild type human TNFα, which is sufficient to induce cell death in 50% of cells in the absence of anti-sera. Cell death is determined by MTS, as described above. Neutralization-ability of sera from TNFα variant-immunized mice are compared to sera obtained from mice immunized with human wild type TNFα.

In vitro studies

Cytotoxicity bioassay using WEHI 164 clone 13- or KYM-1D4-cells: Cytotoxicity-inhibition bioassay using WEHI 164 clone 13- or KYM-1D4-cells.

5 Criteria for choice of best immunogenic constructs

TNF α variants should display minimal cytotoxicity. Immunization of mice with TNF α variants should generate anti-sera with better or equal ability to neutralize human wild type TNF α -mediated cytotoxicity in WEHI- or KYM-1D4 cells as sera obtained
10 from human wild type TNF α -immunized mice.

CLAIMS

1. An immunogenic analogue of a polymeric protein, said polymeric protein consisting of at least 2 monomeric units that are not joined by means of a peptide bond, wherein said analogue
- 5 d) includes substantial fragments of at least 2 monomeric units of said polymeric protein, wherein said substantial fragments are joined via peptide bonds through a peptide linker,
- 10 e) includes at least one MHC Class II binding amino acid sequence that is heterologous to the polymeric protein, and
- 15 f) can be produced as one single expression product from a cell harbouring an expression vector encoding the analogue.
2. The immunogenic analogue according to claim 1 wherein the polymeric protein is a homopolymeric protein.
3. The immunogenic analogue according to claim 1, wherein the polymeric protein is a heteropolymeric protein.
- 20 4. The immunogenic analogue according to any one of the preceding claims, wherein each of the substantial fragments displays a substantial fraction of B-cell epitopes found in the corresponding monomers when being part of the polymeric protein.
- 25 5. The immunogenic analogue according to claim 4, wherein each of the substantial fragments displays essentially all B-cell

epitopes found in the corresponding monomers when being part of the polymeric protein.

6. The immunogenic analogue according to claim 4 or 5, wherein an amino acid sequence derived from a monomeric unit is modified by means of amino acid insertion, substitution, deletion or addition so as to reduce toxicity of the analogue as compared to the multimeric protein and/or so as to introduce the MHC Class II binding amino acid sequence.

7. The immunogenic analogue according to any one of claims 1-6, wherein each of the substantial fractions comprises essentially the complete amino acid sequence of each monomeric unit, either as a continuous sequence or as a sequence including inserts.

8. The immunogenic analogue according to any of the preceding claims, wherein amino acid sequences of all monomeric units of the polymeric protein are represented in the analogue.

9. The immunogenic analogue according to any one of the preceding claims that includes the complete amino acid sequences of the monomers constituting the polymeric protein, either as unbroken sequences or as sequences including inserts.

10. The immunogenic analogue according to any one of the preceding claims, wherein the peptide linker includes or contributes to the presence in the analogue of at least one MHC Class II binding amino acid sequence that is heterologous to the multimeric protein.

11. The immunogenic analogue according to any one of claims 1-9, wherein the peptide linker is free of and does not contribute to the presence of an MHC Class II binding amino acid sequence.

quence in the animal species from where the multimeric protein is derived.

12. The immunogenic analogue according to any one of the preceding claims wherein the MHC Class II binding amino acid sequence binds a majority of MHC Class II molecules from the animal species from where the multimeric protein has been derived.

13. The immunogenic analogue according to any one of the preceding claims, wherein the at least one MHC Class II binding amino acid sequence is selected from a natural T-cell epitope and an artificial MHC-II binding peptide sequence.

14. The immunogenic analogue according to claim 12, wherein the natural T-cell epitope is selected from a Tetanus toxoid epitope such as P2 or P30, a diphtheria toxoid epitope, an influenza virus hemagglutinin epitope, and a *P. falciparum* CS epitope.

15. The immunogenic analogue according to any one of the preceding claims, wherein the 3-dimensional structure of the complete polymeric protein is essentially preserved.

16. The immunogenic analogue according to any one of the preceding claims, wherein the polymeric protein is selected from the group consisting of interleukin 5 (IL5) and tumour necrosis factor α (TNF α)

17. The immunogenic analogue according to claim 16, wherein the polymeric protein is IL5 and wherein the analogue is selected from the group consisting of

- two complete IL5 monomers joined by a peptide linker that includes at least one MHC Class II binding amino acid sequence,
- two complete IL5 monomers joined by an inert peptide
5 linker of which at least one monomer includes a heterologous MHC Class II binding amino acid sequence.

18. The immunogenic analogue according to claim 17 having the linear structure IL-L_m-IL or IL_m-L_i-IL_n or IL-L_i-IL_m or IL-L_i-IL_m or IL_m-L_m-IL_n wherein "IL" is the complete amino acid sequence
10 of monomeric mature IL5, "IL_m" and "IL_n", which may be identical or non-identical, designate a substantially complete amino acid sequence of monomeric mature IL5 including a heterologous MHC Class II binding amino acid sequence, "L_m" is a peptide linker including or contributing to at least one MHC Class II
15 binding amino acid sequence in the analogue, and "L_i" is an inert peptide linker that does not include or contribute to any MHC Class II binding amino acid sequence in the analogue.

19. The immunogenic analogue according to claim 18, wherein L_m, IL_m and IL_n comprise the P2 and/or P30 epitopes of tetanus
20 toxoid or comprises a PADRE, and L_i is a di-glycine linker.

20. The immunogenic analogue according to claim 19, which has the mature amino acid sequence set forth in any one of SEQ ID
NOs: 9, 11, 13 and 15.

21. The immunogenic analogue according to claim 16, wherein
25 the polymeric protein is TNF α and wherein the analogue is selected from the group consisting of

- two or three complete TNF- α monomers joined end-to-end by a peptide linker, wherein at least one peptide linker in-

cludes at least one MHC Class II binding amino acid sequence,

- two or three complete TNF- α monomers joined end-to-end by an inert peptide linker, wherein at least one of the monomers include at least one foreign MHC Class II binding amino acid sequence or wherein at least one foreign MHC Class II binding amino acids sequence is fused to the N- or C-terminal monomer, optionally via an inert linker.

22. An immunogenic analogue of human TNF α , wherein the analogue includes at least one foreign MHC Class II binding amino acid sequence and further has the characteristic of being

- a human TNF α monomer or an analogue according to claim 16, wherein has been inserted or in-substituted at least one foreign MHC Class II binding amino acid sequence into flexible loop 3, and/or
- a human TNF α monomer or an analogue according to claim 16, wherein has been introduced at least one disulfide bridge that stabilises the TNF α monomer 3D structure, and/or
- a human TNF α monomer or an analogue according to claim 16, wherein any one of amino acids 1, 2, 3, 4, 5, 6, 7, 8, and 9 in the amino terminus have been deleted, and/or
- a human TNF α monomer or an analogue according to claim 16, wherein an inserted or in-substituted at least one foreign MHC Class II binding amino acid sequence into loop 1 in an intron position, and/or

- a human TNF α monomer or an analogue according to claim 16, wherein at least one foreign MHC Class II binding amino acid sequence is introduced as part of an artificial stalk region in the N-terminus of human TNF α , and/or
- 5 - a human TNF α monomer or an analogue according to claim 16, wherein at least one foreign MHC Class II binding amino acid sequence is introduced so as to stabilize the monomer structure by increasing the hydrophobicity of the trimeric interaction interface, and/or
- 10 - a human TNF α monomer or an analogue according to claim 16, wherein at least one foreign MHC Class II binding amino acid sequence flanked by glycine residues is inserted or in-substituted in the TNF α amino acid sequence, and/or
- 15 - a human TNF α monomer or an analogue according to claim 16, wherein at least one foreign MHC Class II binding amino acid sequence is inserted or in-substituted in the D-E loop, and/or
- 20 - a human TNF α monomer or an analogue according to claim 16, wherein at least one foreign MHC Class II binding amino acid sequence is inserted or in-substituted between two identical subsequences of human TNF α , and/or
- 25 - a human TNF α monomer or an analogue according to claim 16, wherein at least one salt bridge in human TNF α has been strengthened or substituted with a disulphide bridge, and/or

- a human TNF α monomer or an analogue according to claim 16, wherein solubility and/or stability towards proteolysis is enhanced by introducing mutations that mimic murine TNF α crystalline structure, and/or
 - 5 - a human TNF α monomer or an analogue according to claim 16, wherein potential toxicity is reduced or abolished by introduction of at least one point mutation.
23. An immunogenic analogue according to claim 25 or 26, wherein the amino acid sequence of the analogue is selected
- 10 from the group consisting of SEQ ID NO: 18, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 49, 51, 53, 55, 57, and 59, and any amino acid sequence that only include conservative amino acid changes thereof.
- 15 24. An immunogenic analogue according to any one of the preceding claims which can be expressed as a soluble protein from bacterial cells.
25. A nucleic acid fragment that encodes an immunogenic analogue according to any one of the preceding claims, or a nu-
- 20 cleic acid fragment complementary thereto.
26. The nucleic acid fragment according to claim 25 that is a DNA fragment.
27. The nucleic acid fragment according to claim 25 which comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO: 17, 48, 50, 52, 54, 56, and 58 or a nucleic acid sequence complementary thereto.
- 25

28. A method for down-regulating a polymeric protein in an autologous host, the method comprising effecting presentation to the animal's immune system of an immunogenically effective amount of at least one immunogenic analogue according to any
5 one of claims 1-26.

29. The method according to claim 28, wherein the autologous host is a mammal, such as a human being.

30. The method according to claim 28 or 29, wherein presentation is effected by administering the immunogenic analogue according to any one of claims 1-26 to the autologous host, optionally in admixture with an adjuvant.
10

31. The method according to claim 30, wherein the adjuvant is selected from the group consisting of an immune targeting adjuvant; an immune modulating adjuvant such as a toxin, a cytokine and a mycobacterial derivative; an oil formulation; a polymer; a micelle forming adjuvant; a saponin; an immunostimulating complex matrix (an ISCOM matrix); a particle; DDA; aluminium adjuvants; DNA adjuvants; γ -inulin; and an encapsulating adjuvant.
15

20 32. The method according to any one of claims 28-31, wherein an immunogenically effective amount of analogue is administered to the animal via a route selected from the parenteral route such as the intradermal, the subdermal, and the intramuscular routes; the peritoneal route; the oral route; the buccal route; the sublingual route; the epidural route; the
25 spinal route; the anal route; and the intracranial route.

33. The method according to claim 32, wherein the effective amount is between 0.5 μ g and 2,000 μ g.

34. The method according to claim 32 or 33, which includes at least one administration per year, such as at least 2, at least 3, at least 4, at least 6, and at least 12 administrations per year.
- 5 35. The method according to claim 28, wherein presentation of the analogue to the immune system is effected by introducing nucleic acid(s) encoding the analogue into the animal's cells and thereby obtaining *in vivo* expression by the cells of the nucleic acid(s) introduced.
- 10 36. The method according to claim 35, wherein the nucleic acid(s) introduced is/are selected from naked DNA, DNA formulated with charged or uncharged lipids, DNA formulated in liposomes, DNA included in a viral vector, DNA formulated with a transfection-facilitating protein or polypeptide, DNA formu-
15 lated with a targeting protein or polypeptide, DNA formulated with Calcium precipitating agents, DNA coupled to an inert carrier molecule, DNA encapsulated in chitin or chitosan, and DNA formulated with an adjuvant such as the adjuvants defined in claim 30.
- 20 37. The method according to claim 35 or 36, wherein the nucleic acids are administered intraarterially, intravenously, or by the routes defined in claim 31.
38. The method according to any one of claims 35-37, which includes at least one administration of the nucleic acids per
25 year, such as at least 2, at least 3, at least 4, at least 6, and at least 12 administrations per year.
39. The method according to claim 28, wherein presentation to the immune system is effected by administering a non-patho-

genic microorganism or virus which is carrying a nucleic acid fragment which encodes and expresses the analogue.

40. The method according to claim 39, wherein the virus is a non-virulent pox virus such as a vaccinia virus.

5 41. The method according to claim 40, wherein the microorganism is a bacterium.

42. The method according to any one of claims 39-41, wherein the non-pathogenic microorganism or virus is administered one single time to the animal.

10 43. A composition for inducing production of antibodies against a multimeric protein, the composition comprising

- an immunogenic analogue according to any one of claims 1-26, and

- a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or adjuvant.

15

44. A composition for inducing production of antibodies against a multimeric protein, the composition comprising

- a nucleic acid fragment according to claim 27, and

- a pharmaceutically and immunologically acceptable carrier and/or vehicle and/or adjuvant.

20

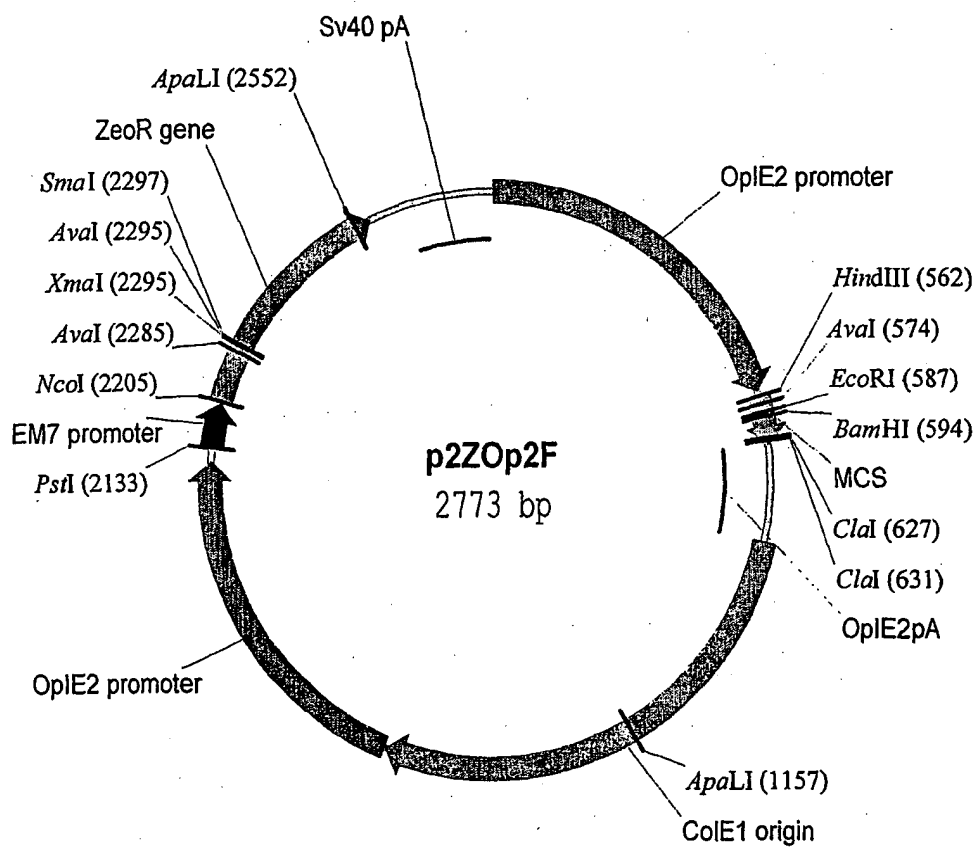
45. The composition according to claim 43 or 43, wherein the analogue is formulated as defined in any one of claims 30 or 31.

46. A method for the preparation of the analogue according to any one of claims 1-26, the method comprising culturing a host cell transformed with the nucleic acid fragment according to claim 27 under conditions that facilitate expression of the
5 nucleic acid fragment of claim 27 and subsequently recovering the analogue as a protein expression product from the culture.

47. The method according to claim 46, wherein the host cell is a bacterial host cell.

48. The method according to claim 47, wherein the analogue is
10 a soluble expression product.

1/1

**Fig. 1**

SEQUENCE LISTING

<110> Pharmexa A/S

<120> NOVEL IMMUNOGENIC MIMETICS OF MULTIMER PROTEINS

<130> P1013DK00

<160> 60

<170> PatentIn version 3.1

<210> 1

<211> 115

<212> PRT

<213> homo sapiens

<400> 1

Ile Pro Thr Glu Ile Pro Thr Ser Ala Leu Val Lys Glu Thr Leu Ala
 1 5 10 15

Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg
 20 25 30

Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr Glu Glu Ile
 35 40 45

Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln Gly Gly Thr
 50 55 60

Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp
 65 70 75 80

Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val Asn Gln Phe
 85 90 95

Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu Trp Ile
 100 105 110

Ile Glu Ser
 115

<210> 2

<211> 45

<212> DNA

<213> Artificial sequence

<220>

<223> Tetanus toxoid P2 epitope

<220>
 <221> CDS
 <222> (1)..(45)
 <223>

<400> 2
 cag tac atc aaa gct aac tcc aaa ttc atc ggc atc acc gaa ctg 45
 Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 1 5 10 15

<210> 3
 <211> 15
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Tetanus toxoid P2 epitope

<400> 3

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 1 5 10 15

<210> 4
 <211> 63
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Tetanus toxoid P30 epitope

<220>
 <221> CDS
 <222> (1)..(63)
 <223>

<400> 4
 ttc aac aac ttc acc gtt tcc ttc tgg ctg cgc gtt cca aaa gtt tcc 48
 Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 1 5 10 15

gct tcc cac ctg gaa 63
 Ala Ser His Leu Glu
 20

<210> 5
 <211> 21
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Tetanus toxoid P30 epitope

<400> 5

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 1 5 10 15

Ala Ser His Leu Glu
 20

<210> 6
 <211> 39
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Pan DR binding peptide (PADRE)

<220>
 <221> CDS
 <222> (1)..(39)
 <223>

<400> 6
 gcc aag ttc gtg gcc gct tgg acc ctg aag gcc gca gct 39
 Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 1 5 10

<210> 7
 <211> 13
 <212> PRT
 <213> Artificial sequence

<220>
 <223> Pan DR binding peptide (PADRE)

<400> 7

Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 1 5 10

<210> 8
 <211> 858
 <212> DNA
 <213> Artificial sequence

<220>
 <223> 2 human IL5 monomers joined by P30 and P2 epitopes

<220>
 <221> CDS
 <222> (1)..(855)
 <223>

<220>
 <221> mat_peptide
 <222> (58)..()
 <223>

<220>

<221> sig_peptide

<222> (1)..(57)

<223>

<400> 8

atg agg atg ctt ctg cat ttg agt ttg ctg gct ctt gga gct gcc tac	48
Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr	
-15 -10 -5	
gtg tat gcc atc ccc aca gaa att ccc aca agt gca ttg gtg aaa gag	96
Val Tyr Ala Ile Pro Thr Glu Ile Pro Thr Ser Ala Leu Val Lys Glu	
-1 1 5 10	
acc ttg gca ctg ctt tct act cat cga act ctg ctg ata gcc aat gag	144
Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu	
15 20 25	
act ctg agg att cct gtt cct gta cat aaa aat cac caa ctg tgc act	192
Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr	
30 35 40 45	
gaa gaa atc ttt cag gga ata ggc aca ctg gag agt caa act gtg caa	240
Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln	
50 55 60	
ggg ggt act gtg gaa aga cta ttc aaa aac ttg tcc tta ata aag aaa	288
Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys	
65 70 75	
tac att gac ggc caa aaa aaa aag tgt gga gaa gaa aga cgg aga gta	336
Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val	
80 85 90	
aac caa ttc cta gac tac ctg caa gag ttt ctt ggt gta atg aac acc	384
Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr	
95 100 105	
gag tgg ata ata gaa agt ttc aac aac ttc acc gtg agc ttc tgg ctg	432
Glu Trp Ile Ile Glu Ser Phe Asn Asn Phe Thr Val Ser Phe Trp Leu	
110 115 120 125	
cgc gtg cct aag gtg agc gcc agc cac ctg gag cag tac atc aag gcc	480
Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Tyr Ile Lys Ala	
130 135 140	
aac tcc aag ttc atc ggc atc acc gag ctg atc ccc aca gaa att ccc	528
Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Ile Pro Thr Glu Ile Pro	
145 150 155	
aca agt gca ttg gtg aaa gag acc ttg gca ctg ctt tct act cat cga	576
Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg	
160 165 170	
act ctg ctg ata gcc aat gag act ctg agg att cct gtt cct gta cat	624
Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His	
175 180 185	

aaa aat cac caa ctg tgc act gaa gaa atc ttt cag gga ata ggc aca 672
 Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr
 190 195 200 205

ctg gag agt caa act gtg caa ggg ggt act gtg gaa aga cta ttc aaa 720
 Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys
 210 215 220

aac ttg tcc tta ata aag aaa tac att gac ggc caa aaa aaa aag tgt 768
 Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys
 225 230 235

gga gaa gaa aga cgg aga gta aac caa ttc cta gac tac ctg caa gag 816
 Gly Glu Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu
 240 245 250

ttt ctt ggt gta atg aac acc gag tgg ata ata gaa agt tga 858
 Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
 255 260 265

<210> 9
 <211> 285
 <212> PRT
 <213> Artificial sequence

<220>
 <223> 2 human IL5 monomers joined by P30 and P2 epitopes

<400> 9

Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

Val Tyr Ala Ile Pro Thr Glu Ile Pro Thr Ser Ala Leu Val Lys Glu
 -1 1 5 10

Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu
 15 20 25

Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr
 30 35 40 45

Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln
 50 55 60

Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys
 65 70 75

Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val
 80 85 90

Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr
 95 100 105

Glu Trp Ile Ile Glu Ser Phe Asn Asn Phe Thr Val Ser Phe Trp Leu
 110 115 120 125

Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Gln Tyr Ile Lys Ala
 130 135 140

Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Ile Pro Thr Glu Ile Pro
 145 150 155

Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg
 160 165 170

Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His
 175 180 185

Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr
 190 195 200 205

Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys
 210 215 220

Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys
 225 230 235

Gly Glu Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu
 240 245 250

Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
 255 260 265

<210> 10
 <211> 858
 <212> DNA
 <213> Artificial sequence

<220>
 <223> 2 human IL5 monomers joined by P2 and P30 epitopes

<220>
 <221> CDS
 <222> (1)..(855)
 <223>

<220>
 <221> mat_peptide

<222> (58)..()

<223>

<220>

<221> sig_peptide

<222> (1)..(57)

<223>

<400> 10

atg agg atg ctt ctg cat ttg agt ttg ctg gct ctt gga gct gcc tac 48
 Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

gtg tat gcc atc ccc aca gaa att ccc aca agt gca ttg gtg aaa gag 96
 Val Tyr Ala Ile Pro Thr Glu Ile Pro Thr Ser Ala Leu Val Lys Glu
 -1 1 5 10

acc ttg gca ctg ctt tct act cat cga act ctg ctg ata gcc aat gag 144
 Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu
 15 20 25

act ctg agg att cct gtt cct gta cat aaa aat cac caa ctg tgc act 192
 Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr
 30 35 40 45

gaa gaa atc ttt cag gga ata ggc aca ctg gag agt caa act gtg caa 240
 Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln
 50 55 60

ggg ggt act gtg gaa aga cta ttc aaa aac ttg tcc tta ata aag aaa 288
 Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys
 65 70 75

tac att gac ggc caa aaa aaa aag tgt gga gaa gaa aga cgg aga gta 336
 Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val
 80 85 90

aac caa ttc cta gac tac ctg caa gag ttt ctt ggt gta atg aac acc 384
 Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr
 95 100 105

gag tgg ata ata gaa agt cag tac atc aag gcc aac tcc aag ttc atc 432
 Glu Trp Ile Ile Glu Ser Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile
 110 115 120 125

ggc atc acc gag ctg ttc aac aac ttc acc gtg agc ttc tgg ctg cgc 480
 Gly Ile Thr Glu Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
 130 135 140

gtg cct aag gtg agc gcc agc cac ctg gag atc ccc aca gaa att ccc 528
 Val Pro Lys Val Ser Ala Ser His Leu Glu Ile Pro Thr Glu Ile Pro
 145 150 155

aca agt gca ttg gtg aaa gag acc ttg gca ctg ctt tct act cat cga 576
 Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg
 160 165 170

act ctg ctg ata gcc aat gag act ctg agg att cct gtt cct gta cat 624
 Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His
 175 180 185

aaa aat cac caa ctg tgc act gaa gaa atc ttt cag gga ata ggc aca 672
 Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr
 190 195 200 205

ctg gag agt caa act gtg caa ggg ggt act gtg gaa aga cta ttc aaa 720
 Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys
 210 215 220

aac ttg tcc tta ata aag aaa tac att gac ggc caa aaa aaa aag tgt 768
 Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys
 225 230 235

gga gaa gaa aga cgg aga gta aac caa ttc cta gac tac ctg caa gag 816
 Gly Glu Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu
 240 245 250

ttt ctt ggt gta atg aac acc gag tgg ata ata gaa agt tga 858
 Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
 255 260 265

<210> 11
 <211> 285
 <212> PRT
 <213> Artificial sequence

<220>
 <223> 2 human IL5 monomers joined by P2 and P30 epitopes

<400> 11

Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

Val Tyr Ala Ile Pro Thr Glu Ile Pro Thr Ser Ala Leu Val Lys Glu
 -1 1 5 10

Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu
 15 20 25

Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr
 30 35 40 45

Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln
 50 55 60

Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys
 65 70 75

Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val
80 85 90

Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr
95 100 105

Glu Trp Ile Ile Glu Ser Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile
110 115 120 125

Gly Ile Thr Glu Leu Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg
130 135 140

Val Pro Lys Val Ser Ala Ser His Leu Glu Ile Pro Thr Glu Ile Pro
145 150 155

Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg
160 165 170

Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His
175 180 185

Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr
190 195 200 205

Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys
210 215 220

Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys
225 230 235

Gly Glu Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu
240 245 250

Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
255 260 265

<210> 12

<211> 864

<212> DNA

<213> Artificial sequence

<220>

<223> Two human IL5 monomers joined by diglycine linker and including terminally positioned P30 and P2 epitopes

<220>

<221> CDS

<222> (1)..(861)

<223>

<220>

<221> misc_feature

<222> (70)..(132)

<223> tetanus toxoid P30 epitope

<220>

<221> misc_feature

<222> (817)..(861)

<223> tetanus toxoid P30 epitope

<220>

<221> mat_peptide

<222> (58)..()

<223>

<220>

<221> sig_peptide

<222> (1)..(57)

<223>

<400> 12

atg	agg	atg	ctt	ctg	cat	ttg	agt	ttg	ctg	gct	ctt	gga	gct	gcc	tac	48
Met	Arg	Met	Leu	Leu	His	Leu	Ser	Leu	Leu	Ala	Leu	Gly	Ala	Ala	Tyr	
				-15					-10					-5		

gtg	tat	gcc	atc	ccc	aca	gaa	ttc	aac	aac	ttc	acc	gtg	agc	ttc	tgg	96
Val	Tyr	Ala	Ile	Pro	Thr	Glu	Phe	Asn	Asn	Phe	Thr	Val	Ser	Phe	Trp	
	-1	1					5					10				

ctg	cgc	gtg	cct	aag	gtg	agc	gcc	agc	cac	ctg	gag	att	ccc	aca	agt	144
Leu	Arg	Val	Pro	Lys	Val	Ser	Ala	Ser	His	Leu	Glu	Ile	Pro	Thr	Ser	
	15					20					25					

gca	ttg	gtg	aaa	gag	acc	ttg	gca	ctg	ctt	tct	act	cat	cga	act	ctg	192
Ala	Leu	Val	Lys	Glu	Thr	Leu	Ala	Leu	Leu	Ser	Thr	His	Arg	Thr	Leu	
30				35					40					45		

ctg	ata	gcc	aat	gag	act	ctg	agg	att	cct	gtt	cct	gta	cat	aaa	aat	240
Leu	Ile	Ala	Asn	Glu	Thr	Leu	Arg	Ile	Pro	Val	Pro	Val	His	Lys	Asn	
			50					55						60		

cac	caa	ctg	tgc	act	gaa	gaa	atc	ttt	cag	gga	ata	ggc	aca	ctg	gag	288
His	Gln	Leu	Cys	Thr	Glu	Glu	Ile	Phe	Gln	Gly	Ile	Gly	Thr	Leu	Glu	
		65					70					75				

agt	caa	act	gtg	caa	ggg	ggt	act	gtg	gaa	aga	cta	ttc	aaa	aac	ttg	336
Ser	Gln	Thr	Val	Gln	Gly	Gly	Thr	Val	Glu	Arg	Leu	Phe	Lys	Asn	Leu	
		80				85						90				

ttc	tta	ata	aag	aaa	tac	att	gac	ggc	caa	aaa	aaa	aag	tgt	gga	gaa	384
Ser	Leu	Ile	Lys	Lys	Tyr	Ile	Asp	Gly	Gln	Lys	Lys	Lys	Cys	Gly	Glu	
	95					100					105					

gaa aga cgg aga gta aac caa ttc cta gac tac ctg caa gag ttt ctt 432
 Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu
 110 115 120 125
 ggt gta atg aac acc gag tgg ata ata gaa agt ggc ggt atc ccc aca 480
 Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser Gly Gly Ile Pro Thr
 130 135 140
 gaa att ccc aca agt gca ttg gtg aaa gag acc ttg gca ctg ctt tct 528
 Glu Ile Pro Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser
 145 150 155
 act cat cga act ctg ctg ata gcc aat gag act ctg agg att cct gtt 576
 Thr His Arg Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val
 160 165 170
 cct gta cat aaa aat cac caa ctg tgc act gaa gaa atc ttt cag gga 624
 Pro Val His Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly
 175 180 185
 ata ggc aca ctg gag agt caa act gtg caa ggg ggt act gtg gaa aga 672
 Ile Gly Thr Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg
 190 195 200 205
 cta ttc aaa aac ttg tcc tta ata aag aaa tac att gac ggc caa aaa 720
 Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys
 210 215 220
 aaa aag tgt gga gaa gaa aga cgg aga gta aac caa ttc cta gac tac 768
 Lys Lys Cys Gly Glu Glu Arg Arg Val Asn Gln Phe Leu Asp Tyr
 225 230 235
 ctg caa gag ttt ctt ggt gta atg aac acc gag tgg ata ata gaa agt 816
 Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
 240 245 250
 cag tac atc aag gcc aac tcc aag ttc atc ggc atc acc gag ctg tga 864
 Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 255 260 265

<210> 13

<211> 287

<212> PRT

<213> Artificial sequence

<220>

<223> Two human IL5 monomers joined by diglycine linker and including t
 erminally positioned P30 and P2 epitopes

<400> 13

Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

Val Tyr Ala Ile Pro Thr Glu Phe Asn Asn Phe Thr Val Ser Phe Trp
 -1 1 5 10

Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu Ile Pro Thr Ser
15 20 25

Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu
30 35 40 45

Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His Lys Asn
50 55 60

His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu
65 70 75

Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu
80 85 90

Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu
95 100 105

Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu
110 115 120 125

Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser Gly Gly Ile Pro Thr
130 135 140

Glu Ile Pro Thr Ser Ala Leu Val Lys Glu Thr Leu Ala Leu Leu Ser
145 150 155

Thr His Arg Thr Leu Leu Ile Ala Asn Glu Thr Leu Arg Ile Pro Val
160 165 170

Pro Val His Lys Asn His Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly
175 180 185

Ile Gly Thr Leu Glu Ser Gln Thr Val Gln Gly Gly Thr Val Glu Arg
190 195 200 205

Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys
210 215 220

Lys Lys Cys Gly Glu Glu Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr
225 230 235

Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu Trp Ile Ile Glu Ser
240 245 250

Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 255 260 265

<210> 14
 <211> 864
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Two human IL5 monomers joined by a di-glycine linker and including terminally positioned tetanus toxoid P2 and P30 epitopes

<220>
 <221> CDS
 <222> (1)..(861)
 <223>

<220>
 <221> misc_feature
 <222> (70)..(114)
 <223> tetanus toxoid P2 epitope

<220>
 <221> misc_feature
 <222> (817)..(861)
 <223> tetanus toxoid P2 epitope

<220>
 <221> sig_peptide
 <222> (1)..(57)
 <223>

<220>
 <221> mat_peptide
 <222> (58)..()
 <223>

<400> 14
 atg agg atg ctt ctg cat ttg agt ttg ctg gct ctt gga gct gcc tac 48
 Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

gtg tat gcc atc ccc aca gaa cag tac atc aag gcc aac tcc aag ttc 96
 Val Tyr Ala Ile Pro Thr Glu Gln Tyr Ile Lys Ala Asn Ser Lys Phe
 -1 1 5 10

atc ggc atc acc gag ctg att ccc aca agt gca ttg gtg aaa gag acc 144
 Ile Gly Ile Thr Glu Leu Ile Pro Thr Ser Ala Leu Val Lys Glu Thr
 15 20 25

ttg gca ctg ctt tct act cat cga act ctg ctg ata gcc aat gag act 192
 Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu Thr
 30 35 40 45

ctg agg att cct gtt cct gta cat aaa aat cac caa ctg tgc act gaa Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr Glu	240
50 55 60	
gaa atc ttt cag gga ata ggc aca ctg gag agt caa act gtg caa ggg Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln Gly	288
65 70 75	
ggt act gtg gaa aga cta ttc aaa aac ttg tcc tta ata aag aaa tac Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys Tyr	336
80 85 90	
att gac ggc caa aaa aaa aag tgt gga gaa gaa aga cgg aga gta aac Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val Asn	384
95 100 105	
caa ttc cta gac tac ctg caa gag ttt ctt ggt gta atg aac acc gag Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu	432
110 115 120 125	
tgg ata ata gaa agt ggc ggt atc ccc aca gaa att ccc aca agt gca Trp Ile Ile Glu Ser Gly Gly Ile Pro Thr Glu Ile Pro Thr Ser Ala	480
130 135 140	
ttg gtg aaa gag acc ttg gca ctg ctt tct act cat cga act ctg ctg Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu	528
145 150 155	
ata gcc aat gag act ctg agg att cct gtt cct gta cat aaa aat cac Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His	576
160 165 170	
caa ctg tgc act gaa gaa atc ttt cag gga ata ggc aca ctg gag agt Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser	624
175 180 185	
caa act gtg caa ggg ggt act gtg gaa aga cta ttc aaa aac ttg tcc Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser	672
190 195 200 205	
tta ata aag aaa tac att gac ggc caa aaa aaa aag tgt gga gaa gaa Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu	720
210 215 220	
aga cgg aga gta aac caa ttc cta gac tac ctg caa gag ttt ctt ggt Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly	768
225 230 235	
gta atg aac acc gag tgg ata ata gaa agt ttc aac aac ttc acc gtg Val Met Asn Thr Glu Trp Ile Ile Glu Ser Phe Asn Asn Phe Thr Val	816
240 245 250	
agc ttc tgg ctg cgc gtg cct aag gtg agc gcc agc cac ctg gag tga Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu	864
255 260 265	

<210> 15

<211> 287

<212> PRT

<213> Artificial sequence

<220>

<223> Two human IL5 monomers joined by a di-glycine linker and including terminally positioned tetanus toxoid P2 and P30 epitopes

<400> 15

Met Arg Met Leu Leu His Leu Ser Leu Leu Ala Leu Gly Ala Ala Tyr
 -15 -10 -5

Val Tyr Ala Ile Pro Thr Glu Gln Tyr Ile Lys Ala Asn Ser Lys Phe
 -1 1 5 10

Ile Gly Ile Thr Glu Leu Ile Pro Thr Ser Ala Leu Val Lys Glu Thr
 15 20 25

Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu Ile Ala Asn Glu Thr
 30 35 40 45

Leu Arg Ile Pro Val Pro Val His Lys Asn His Gln Leu Cys Thr Glu
 50 55 60

Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser Gln Thr Val Gln Gly
 65 70 75

Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser Leu Ile Lys Lys Tyr
 80 85 90

Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu Arg Arg Arg Val Asn
 95 100 105

Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly Val Met Asn Thr Glu
 110 115 120 125

Trp Ile Ile Glu Ser Gly Gly Ile Pro Thr Glu Ile Pro Thr Ser Ala
 130 135 140

Leu Val Lys Glu Thr Leu Ala Leu Leu Ser Thr His Arg Thr Leu Leu
 145 150 155

Ile Ala Asn Glu Thr Leu Arg Ile Pro Val Pro Val His Lys Asn His
 160 165 170

Gln Leu Cys Thr Glu Glu Ile Phe Gln Gly Ile Gly Thr Leu Glu Ser
 175 180 185

Gln Thr Val Gln Gly Gly Thr Val Glu Arg Leu Phe Lys Asn Leu Ser
190 195 200 205

Leu Ile Lys Lys Tyr Ile Asp Gly Gln Lys Lys Lys Cys Gly Glu Glu
210 215 220

Arg Arg Arg Val Asn Gln Phe Leu Asp Tyr Leu Gln Glu Phe Leu Gly
225 230 235

Val Met Asn Thr Glu Trp Ile Ile Glu Ser Phe Asn Asn Phe Thr Val
240 245 250

Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu Glu
255 260 265

<210> 16
<211> 477
<212> DNA
<213> Artificial sequence

<220>
<223> Human wt TNF (codons optimised)

<220>
<221> CDS
<222> (1)..(477)
<223>

<220>
<221> misc_feature
<222> (4)..(474)
<223> Mature TNF sequence

<400> 16
atg gtg cgc tca agc tcg cgc acg ccg agt gac aaa cca gta gct cat 48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

gtt gtg gcc aac cct cag gcg gaa ggc cag ctc caa tgg tta aat cgt 96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
20 25 30

cgc gcg aac gcc ctg ctg gcg aac ggc gtg gaa ctg cgt gat aac cag 144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
35 40 45

ctg gtg gtc ccc agc gag ggg ctg tat ctg atc tat tca cag gtg ttg 192
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
50 55 60


```

ttt aag ggt cag ggt tgt ccg agc acc cac gtt ctg ctg acg cat acc      240
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65                               70                               75                               80

att tct cgt att gct gta tct tat caa act aaa gtc aat tta ctt tcg      288
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
                               85                               90                               95

gcg atc aaa tcc ccg tgc caa cgt gag acc cct gaa gga gcg gaa gcc      336
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
                               100                              105                              110

aaa cct tgg tac gaa ccg atc tat ctg ggg ggc gtt ttt cag ctc gaa      384
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
                               115                              120                              125

aaa ggt gat cgg ctg agc gcc gaa att aat cgc ccg gac tac ctt gat      432
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
                               130                              135                              140

ttc gca gag tcc ggt cag gtc tac ttc ggc att atc gca ttg taa      477
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145                               150                               155

```

<210> 17

<211> 158

<212> PRT

<213> Artificial sequence

<220>

<223> Human wt TNF (codons optimised)

<400> 17

```

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1                               5                               10\                               15

```

```

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
                20                25                30

```

```

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
                35                40                45

```

```

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
                50                55                60

```

```

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65                               70                               75                               80

```

```

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
                85                90                95

```

18

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 145 150 155

<210> 18
 <211> 170
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108

<220>
 <221> MISC_FEATURE
 <222> (122)..(170)
 <223> hTNF amino acids 109-157

<400> 18

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

19

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Ala Lys Pro Trp Tyr
115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
165 170

<210> 19
<211> 39
<212> DNA
<213> Artificial sequence

<220>
<223> Pan DR binding peptide (PADRE)

<220>
<221> CDS
<222> (1)..(39)
<223>

<400> 19
gcg aag ttc gtt gca gct tgg acc ctg aag gcc gct gca
Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
1 5 10

39

<210> 20
<211> 13
<212> PRT
<213> Artificial sequence

<220>
<223> Pan DR binding peptide (PADRE)

<400> 20

Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 1 5 10

<210> 21
 <211> 1437
 <212> DNA
 <213> Artificial sequence

<220>
 <223> Monomeric mimic of trimeric human TNF

<220>
 <221> CDS
 <222> (1)..(1437)
 <223>

<220>
 <221> mutation
 <222> (475)..(483)
 <223> coding sequence for tri-glycine linker

<220>
 <221> mutation
 <222> (955)..(963)
 <223> coding sequence for tri-glycine linker

<400> 21
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15
 gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30
 cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag 144
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45
 ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg 192
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60
 ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc 240
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80
 atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc 288
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95
 gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc 336
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 115 120 125	384
aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 130 135 140	432
ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 145 150 155 160	480
gga gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 165 170 175	528
gtc gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 180 185 190	576
cgg gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 195 200 205	624
ctt gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 210 215 220	672
ttt aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 225 230 235 240	720
att tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 245 250 255	768
gct att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 260 265 270	816
aaa cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 275 280 285	864
aaa ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 290 295 300	912
ttt gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 305 310 315 320	960
ggg gtt cgt tct tct tct cgt acg ccg tct gat aag ccg gtt gcg cac Gly Val Arg Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 325 330 335	1008
gtt gtt gcg aac ccg cag gcg gag ggg caa ttg cag tgg ttg aat cgt Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 340 345 350	1056

cgt gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg cgt gat aac caa 1104
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 355 360 365
 ttg gtt gtt ccg tct gag ggg ttg tac ttg ata tat tct cag gtt ttg 1152
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 370 375 380
 ttc aaa ggg caa ggg tgc ccg tct acg cat gtt ttg ttg acg cac acg 1200
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 385 390 395 400
 ata tct cgt ata gcg gtt tct tac cag acg aag gtt aat ttg ttg tct 1248
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 405 410 415
 gcg ata aaa tct ccg tgt caa cgt gaa acg ccg gaa ggg gcg gag gcg 1296
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 420 425 430
 aag ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt ttt cag ttg gaa 1344
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 435 440 445
 aaa ggg gat cgt ttg tct gcg gag ata aac cgt ccg gac tat ttg gat 1392
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 450 455 460
 ttc gcg gaa tct ggg caa gtt tac ttt ggg ata ata gcg ctg taa 1437
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 465 470 475

<210> 22

<211> 478

<212> PRT

<213> Artificial sequence

<220>

<223> Monomeric mimic of trimeric human TNF

<400> 22

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
145 150 155 160

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
165 170 175

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
180 185 190

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
195 200 205

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
210 215 220

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
225 230 235 240

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
245 250 255

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
260 265 270

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
275 280 285

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
290 295 300

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 305 310 315 320
 Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 325 330 335
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 340 345 350
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 355 360 365
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 370 375 380
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 385 390 395 400
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 405 410 415
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 420 425 430
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 435 440 445
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 450 455 460
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 465 470 475

<210> 23
 <211> 170
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE

<220>
 <221> MUTAGEN
 <222> (107)..(119)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(106)
 <223> hTNF amino acids 1-106

<220>
 <221> MISC_FEATURE
 <222> (120)..(170)
 <223> hTNF amino acids 107-157

<400> 23

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Ala Lys Phe Val Ala Ala
 100 105 110

Trp Thr Leu Lys Ala Ala Ala Glu Gly Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 24
 <211> 170
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE

<220>
 <221> MUTAGEN
 <222> (108)..(120)
 <223>

<220>
 <221> MISC_FEATURE
 <222> (1)..(107)
 <223> hTNF amino acids 1-107

<220>
 <221> MISC_FEATURE
 <222> (121)..(170)
 <223> hTNF amino acids 108-157

<400> 24

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Ala Lys Phe Val Ala
 100 105 110

Ala Trp Thr Leu Lys Ala Ala Ala Gly Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 25
 <211> 169
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with 12 amino acids of PADRE inserted

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223>

<220>
 <221> MISC_FEATURE
 <222> (1)..(109)
 <223> hTNF amino acids 1-109

<220>
 <221> MISC_FEATURE
 <222> (122)..(169)
 <223> hTNF amino acids 110-157

<400> 25

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Ala Lys Pro Trp Tyr Glu
115 120 125

Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
130 135 140

Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly
145 150 155 160

Gln Val Tyr Phe Gly Ile Ile Ala Leu
165

<210> 26
<211> 167
<212> PRT
<213> Artificial sequence

<220>
<223> hTNF with PADRE substituted in

<220>
<221> MUTAGEN
<222> (109)..(121)
<223> PADRE

<220>
<221> MISC_FEATURE
<222> (1)..(109)
<223> hTNF amino acids 1-109

<220>
<221> MISC_FEATURE
<222> (121)..(167)
<223> hTNF amino acids 111-157

<400> 26

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Lys Pro Trp Tyr Glu Pro Ile
 115 120 125

Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala
 130 135 140

Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val
 145 150 155 160

Tyr Phe Gly Ile Ile Ala Leu
 165

<210> 27
 <211> 165
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with in-substituted PADRE

<220>
 <221> MUTAGEN
 <222> (107)..(119)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(106)
 <223> hTNF amino acids 1-106

<220>
 <221> MISC_FEATURE

<222> (119)..(165)

<223> hTNF amino acids 111-157

<400> 27

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Ala Lys Phe Val Ala Ala
 100 105 110

Trp Thr Leu Lys Ala Ala Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu
 115 120 125

Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile
 130 135 140

Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
 145 150 155 160

Gly Ile Ile Ala Leu
 165

<210> 28

<211> 173

<212> PRT

<213> Artificial sequence

<220>

<223> hTNF with C-terminal tri-glycine linker and PADRE

<220>

<221> MISC_FEATURE
 <222> (1)..(157)
 <223> hTNF

<220>
 <221> MUTAGEN
 <222> (158)..(160)
 <223> tri-glycine linker peptide

<220>
 <221> MUTAGEN
 <222> (161)..(173)
 <223> PADRE

<400> 28

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 115 120 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
 130 135 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly Gly
 145 150 155 160

Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 165 170

<210> 29
 <211> 170
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE and additional disulfide bridge

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108

<220>
 <221> MUTAGEN
 <222> (124)..(124)
 <223> Ala to Cys mutation

<220>
 <221> DISULFID
 <222> (67)..(124)
 <223>

<220>
 <221> MUTAGEN
 <222> (67)..(67)
 <223> Leu to Cys mutation

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (122)..(170)
 <223> hTNF amino acids 109-157

<400> 29

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Cys Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Cys Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 30

<211> 170

<212> PRT

<213> Artificial sequence.

<220>

<223> hTNF with inserted PADRE and additional disulphide bridge

<220>

<221> MISC_FEATURE

<222> (1)..(108)

<223> hTNF residues 1-108 with one mutation

<220>

<221> MISC_FEATURE

<222> (122)..(170)

<223> hTNF residues 109-157 with one mutation

<220>

<221> MUTAGEN

<222> (96)..(96)

<223> Ala to Cys mutation

<220>
 <221> MUTAGEN
 <222> (131)..(131)
 <223> Ile to Cys mutation

<220>
 <221> DISULFID
 <222> (96)..(131)
 <223>

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<400> 30

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Cys
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Cys Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 31
 <211> 162
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF truncate with inserted PADRE

<220>
 <221> MUTAGEN
 <222> (101)..(113)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(100)
 <223> hTNF amino acids 9-108

<220>
 <221> MISC_FEATURE
 <222> (114)..(160)
 <223> hTNF amino acids 109-155

<220>
 <221> MISC_FEATURE
 <222> (114)..(162)
 <223> hTNF amino acids 109-157

<400> 31

Ser Asp Lys Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly
 1 5 10 15

Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly
 20 25 30

Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr
 35 40 45

Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
 50 55 60

His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln
 65 70 75 80

Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu
 85 90 95

Thr Pro Glu Gly Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala
 100 105 110

Ala Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
 115 120 125

Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
 130 135 140

Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 145 150 155 160

Ala Leu

<210> 32
 <211> 169
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with PADRE inserted

<220>
 <221> MUTAGEN
 <222> (18)..(30)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(18)
 <223> hTNF amino acids 1-18

<220>
 <221> MISC_FEATURE
 <222> (31)..(169)
 <223> hTNF amino acids 19-157

<400> 32

Val Arg Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Asn Pro
 20 25 30

Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu
 35 40 45

Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser
 50 55 60

Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly
 65 70 75 80

Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala
 85 90 95

Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro
 100 105 110

Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu
 115 120 125

Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu
 130 135 140

Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly
 145 150 155 160

Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165

<210> 33
 <211> 165
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with in-substituted PADRE

<220>
 <221> MUTAGEN
 <222> (18)..(30)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(18)
 <223> hTNF amino acids 1-18

<220>
 <221> MISC_FEATURE

<222> (30)..(165)

<223> hTNF amino acids 22-157

<400> 33

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Gly
 20 25 30

Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly
 35 40 45

Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr
 50 55 60

Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr
 65 70 75 80

His Val Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln
 85 90 95

Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu
 100 105 110

Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu
 115 120 125

Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile
 130 135 140

Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe
 145 150 155 160

Gly Ile Ile Ala Leu
 165

<210> 34

<211> 170

<212> PRT

<213> Artificial sequence

<220>

<223> hTNF with added artificial stalk region and inserted PADRE

<220>

<221> MUTAGEN
 <222> (2)..(14)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (15)..(170)
 <223> hTNF amino acids 2-157

<400> 34

Met Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Arg Ser
 1 5 10 15

Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val Val Ala Asn
 20 25 30

Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg Ala Asn Ala
 35 40 45

Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu Val Val Pro
 50 55 60

Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe Lys Gly Gln
 65 70 75 80

Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile Ser Arg Ile
 85 90 95

Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser
 100 105 110

Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 35
 <211> 170

<212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE and single stabilising mutation

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108

<220>
 <221> MISC_FEATURE
 <222> (122)..(170)
 <223> hTNF amino acids 109-157 with one mutation

<220>
 <221> MUTAGEN
 <222> (170)..(170)
 <223> Leu to Phe mutation

<400> 35

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Phe
 165 170

<210> 36
 <211> 170
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE and one single mutation

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108 with one single mutation

<220>
 <221> MISC_FEATURE
 <222> (122)..(170)
 <223> hTNF amino acids 109-157

<220>
 <221> MUTAGEN
 <222> (49)..(49)
 <223> Val to Phe mutation

<400> 36

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Phe Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Glu Ala Lys Pro Trp Tyr
 115 120 125

Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg
 130 135 140

Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser
 145 150 155 160

Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170

<210> 37
 <211> 174
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted glycine-linked PADRE

<220>
 <221> MUTAGEN
 <222> (111)..(123)
 <223> PADRE

<220>
 <221> MUTAGEN
 <222> (109)..(110)
 <223> di-glycine linker

<220>
 <221> MUTAGEN
 <222> (124)..(125)

<223> di-glycine linker

<220>

<221> MISC_FEATURE

<222> (1)..(108)

<223> hTNF amino acids 1-108

<220>

<221> MISC_FEATURE

<222> (126)..(174)

<223> hTNF amino acids 109-157

<400> 37

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Gly Gly Ala Lys
100 105 110

Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Gly Gly Ala Glu Ala
115 120 125

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
130 135 140

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
145 150 155 160

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
165 170

<210> 38
<211> 167
<212> PRT
<213> Artificial sequence

<220>
<223> hTNF with in-substituted PADRE

<220>
<221> MUTAGEN
<222> (84)..(96)
<223> PADRE

<220>
<221> MISC_FEATURE
<222> (1)..(84)
<223> hTNF amino acids 1-84

<220>
<221> MISC_FEATURE
<222> (97)..(167)
<223> hTNF amino acids 87-157

<400> 38

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
85 90 95

Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln
100 105 110

45

Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile
 115 120 125

Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala
 130 135 140

Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val
 145 150 155 160

Tyr Phe Gly Ile Ile Ala Leu
 165

<210> 39
 <211> 157
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with in-substituted PADRE

<220>
 <221> MUTAGEN
 <222> (133)..(147)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(132)
 <223> hTNF amino acids 1-132

<220>
 <221> MISC_FEATURE
 <222> (148)..(157)
 <223> hTNF amino acids 148-157

<400> 39

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
115 120 125

Gly Asp Arg Leu Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala
130 135 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145 150 155

<210> 40

<211> 160

<212> PRT

<213> Artificial sequence

<220>

<223> hTNF with in-substituted PADRE

<220>

<221> MUTAGEN

<222> (136)..(148)

<223> PADRE

<220>

<221> MISC_FEATURE

<222> (1)..(135)

<223> hTNF amino acids 1-135

<220>

<221> MISC_FEATURE

<222> (149)..(160)

<223> hTNF amino acids 146-157

<400> 40

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 115 120 125

Gly Asp Arg Leu Ser Ala Glu Ala Lys Phe Val Ala Ala Trp Thr Leu
 130 135 140

Lys Ala Ala Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 145 150 155 160

<210> 41
 <211> 157
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with insubstituted PADRE

<220>
 <221> MUTAGEN
 <222> (64)..(76)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(63)
 <223> hTNF amino acids 1-63

<220>
 <221> MISC_FEATURE
 <222> (77)..(157)
 <223> hTNF amino acids 77-157

<400> 41

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Ala
50 55 60

Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
115 120 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
130 135 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145 150 155

<210> 42

<211> 157

<212> PRT

<213> Artificial sequence

<220>

<223> hTNF with in-substituted PADRE

<220>

<221> MUTAGEN

<222> (72)..(84)

<223>

<220>

<221> MISC_FEATURE

<222> (1)..(71)

<223> hTNF amino acids 1-71

<220>

<221> MISC_FEATURE

<222> (85)..(157)

<223> hTNF amino acids 85-157

<400> 42

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
50 55 60

Lys Gly Gln Gly Cys Pro Ser Ala Lys Phe Val Ala Ala Trp Thr Leu
65 70 75 80

Lys Ala Ala Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
115 120 125

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
130 135 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
145 150 155

<210> 43

<211> 157

<212> PRT

<213> Artificial sequence

<220>

<223> hTNF with insubstituted PADRE

<220>
 <221> MUTAGEN
 <222> (127)..(139)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(126)
 <223> hTNF amino acids 1-126

<220>
 <221> MISC_FEATURE
 <222> (140)..(157)
 <223> hTNF amino acids 140-157

<400> 43

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 100 105 110

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Ala Lys
 115 120 125

Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala Asp Tyr Leu Asp Phe
 130 135 140

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 145 150 155

<210> 44
 <211> 176
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted peptide and duplication of 6 amino acids

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108

<220>
 <221> MISC_FEATURE
 <222> (122)..(176)
 <223> hTNF amino acids 103-157

<400> 44

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
 100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Arg Glu Thr Pro Glu Gly Ala
 115 120 125

Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln
 130 135 140

Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr
 145 150 155 160

Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
 165 170 175

<210> 45
 <211> 174
 <212> PRT
 <213> Artificial sequence

<220>
 <223> hTNF with inserted PADRE and duplication of 4 amino acids

<220>
 <221> MUTAGEN
 <222> (109)..(121)
 <223> PADRE

<220>
 <221> MISC_FEATURE
 <222> (1)..(108)
 <223> hTNF amino acids 1-108

<220>
 <221> MISC_FEATURE
 <222> (122)..(174)
 <223> hTNF amino acids 105-157

<400> 45

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 1 5 10 15

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 20 25 30

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 35 40 45

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 50 55 60

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
65 70 75 80

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
85 90 95

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Lys Phe Val
100 105 110

Ala Ala Trp Thr Leu Lys Ala Ala Ala Thr Pro Glu Gly Ala Glu Ala
115 120 125

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
130 135 140

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
145 150 155 160

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu
165 170

<210> 46
<211> 194
<212> PRT
<213> Artificial sequence

<220>
<223> hTNF with inserted tetanus toxoid P2 and P30 epitopes

<220>
<221> MUTAGEN
<222> (110)..(124)
<223> Tetanus toxoid P2 epitope (SEQ ID NO: 2)

<220>
<221> MUTAGEN
<222> (125)..(145)
<223> Tetanus toxoid P30 epitope (SEQ ID NO: 3)

<220>
<221> MISC_FEATURE
<222> (2)..(109)
<223> hTNF amino acids 1-108

<220>
<221> MISC_FEATURE
<222> (146)..(194)
<223> hTNF amino acids 109-157

<400> 46

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Gln Tyr Ile
100 105 110

Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu Phe Asn Asn Phe
115 120 125

Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His Leu
130 135 140

Glu Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
145 150 155 160

Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
165 170 175

Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
180 185 190

Ala Leu

<210> 47

<211> 194

<212> PRT

<213> Artificial sequence

<220>
 <223> hTNF with inserted tetanus toxoid P2 and P30 epitopes

<220>
 <221> MUTAGEN
 <222> (110)..(130)
 <223> Tetanus toxoid P30 epitope

<220>
 <221> MUTAGEN
 <222> (131)..(145)
 <223> Tetanus toxoid P2 epitope

<220>
 <221> MISC_FEATURE
 <222> (2)..(109)
 <223> hTNF amino acids 1-108

<220>
 <221> MISC_FEATURE
 <222> (146)..(194)
 <223> hTNF amino acids 109-157

<400> 47

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Phe Asn Asn
 100 105 110

Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
115 120 125

Leu Glu Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu
130 135 140

Leu Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
145 150 155 160

Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
165 170 175

Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
180 185 190

Ala Leu

<210> 48
<211> 1545
<212> DNA
<213> Artificial sequence

<220>
<223> 3 hTNF sequences joined by glycine linkers and tetanus toxoid P2
and P30 epitopes

<220>
<221> misc_feature
<222> (475)..(483)
<223> Tri-glycine linker

<220>
<221> misc_feature
<222> (4)..(474)
<223> hTNF

<220>
<221> misc_feature
<222> (1072)..(1542)
<223> hTNF

<220>
<221> misc_feature
<222> (529)..(999)
<223> hTNF

<220>
<221> misc_feature

<222> (1000)..(1008)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (1009)..(1071)
 <223> Tetanus toxoid P30 epitope

<220>
 <221> CDS
 <222> (1)..(1545)
 <223>

<220>
 <221> misc_feature
 <222> (484)..(528)
 <223> Tetanus toxoid P2 epitope

<400> 48
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag 144
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg 192
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc 240
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc 288
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc 336
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag 384
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac 432
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 145 150 155 160	480
gga cag tac atc aaa gct aac tcc aaa ttc atc ggc atc acc gaa ctg Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu 165 170 175	528
gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat gtc Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val 180 185 190	576
gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg cgg Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg 195 200 205	624
gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa ctt Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu 210 215 220	672
gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt ttt Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe 225 230 235 240	720
aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca att Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile 245 250 255	768
tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc gct Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala 260 265 270	816
att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct aaa Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys 275 280 285	864
cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa aaa Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys 290 295 300	912
ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac ttt Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe 305 310 315 320	960
gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt ggg Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly Gly 325 330 335	1008
ttc aac aac ttc acc gtt tcc ttc tgg ctg cgc gtt cca aaa gtt tcc Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser 340 345 350	1056
gct tcc cac ctg gaa gtt cgt tct tct tct cgt acg ccg tct gat aag Ala Ser His Leu Glu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys 355 360 365	1104
ccg gtt gcg cac gtt gtt gcg aac ccg cag gcg gag ggg caa ttg cag Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln 370 375 380	1152

tgg ttg aat cgt cgt gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg 1200
 Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu
 385 390 395 400
 cgt gat aac caa ttg gtt gtt ccg tct gag ggg ttg tac ttg ata tat 1248
 Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr
 405 410 415
 tct cag gtt ttg ttc aaa ggg caa ggg tgc ccg tct acg cat gtt ttg 1296
 Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu
 420 425 430
 ttg acg cac acg ata tct cgt ata gcg gtt tct tac cag acg aag gtt 1344
 Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val
 435 440 445
 aat ttg ttg tct gcg ata aaa tct ccg tgt caa cgt gaa acg ccg gaa 1392
 Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
 450 455 460
 ggg gcg gag gcg aag ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt 1440
 Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
 465 470 475 480
 ttt cag ttg gaa aaa ggg gat cgt ttg tct gcg gag ata aac cgt ccg 1488
 Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
 485 490 495
 gac tat ttg gat ttc gcg gaa tct ggg caa gtt tac ttt ggg ata ata 1536
 Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 500 505 510
 gcg ctg taa 1545
 Ala Leu

<210> 49

<211> 514

<212> PRT

<213> Artificial sequence

<220>

<223> 3 hTNF sequences joined by glycine linkers and tetanus toxoid P2
 and P30 epitopes

<400> 49

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160

Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 165 170 175

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 180 185 190

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 195 200 205

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 210 215 220

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 225 230 235 240

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 245 250 255

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 260 265 270

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 275 280 285

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 290 295 300

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
 305 310 315 320

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly Gly
 325 330 335

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 340 345 350

Ala Ser His Leu Glu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys
 355 360 365

Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln
 370 375 380

Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu
 385 390 395 400

Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr
 405 410 415

Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu
 420 425 430

Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val
 435 440 445

Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
 450 455 460

Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
 465 470 475 480

Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
 485 490 495

Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 500 505 510

Ala Leu

<210> 50
<211> 1545
<212> DNA
<213> Artificial sequence

<220>
<223> 3 hTNF monomers joined by tri-glycine linkers and tetanus toxoid
P2 and P30 epitopes

<220>
<221> CDS
<222> (1)..(1545)
<223>

<220>
<221> misc_feature
<222> (1072)..(1542)
<223> hTNF

<220>
<221> misc_feature
<222> (547)..(1017)
<223> hTNF

<220>
<221> misc_feature
<222> (475)..(483)
<223> Tri-glycine linker

<220>
<221> misc_feature
<222> (484)..(546)
<223> Tetanus toxoid P30 epitope

<220>
<221> misc_feature
<222> (1018)..(1026)
<223> Tri-glycine linker

<220>
<221> misc_feature
<222> (1027)..(1071)
<223> Tetanus toxoid P2 epitope

<220>
<221> misc_feature
<222> (4)..(474)
<223> hTNF

<400> 50
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15
 gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30
 cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag 144
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45
 ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg 192
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60
 ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc 240
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80
 atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc 288
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95
 gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc 336
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110
 aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag 384
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125
 aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac 432
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140
 ttc gcc gag agc ggc cag gtgtac ttc ggc atc atc gcc ctg ggt ggc 480
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160
 gga ttc aac aac ttc acc gtt tcc ttc tgg ctg cgc gtt cca aaa gtt 528
 Gly Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val
 165 170 175
 tcc gct tcc cac ctg gaa gtc cgg tcc tcc tcc cgg aca cca tcc gac 576
 Ser Ala Ser His Leu Glu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp
 180 185 190
 aaa cca gtc gct cat gtc gtc gct aat cca caa gct gaa ggt caa ctt 624
 Lys Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu
 195 200 205
 caa tgg ctt aat cgg cgg gct aat gct ctt ctt gct aat ggt gtc gaa 672
 Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
 210 215 220

ctt cgg gac aat caa ctt gtc gtc cca tcc gaa ggt ctt tat ctt att Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile 225 230 235 240	720
tat tcc caa gtc ctt ttt aaa ggt caa ggt tgt cca tcc aca cat gtc Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val 245 250 255	768
ctt ctt aca cat aca att tcc cgg att gct gtc tcc tat caa aca aaa Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys 260 265 270	816
gtc aat ctt ctt tcc gct att aaa tcc cca tgt caa cgg gaa aca cca Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro 275 280 285	864
gaa ggt gct gaa gct aaa cct tgg tat gaa cca att tat ctt ggt ggt Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly 290 295 300	912
gtc ttt caa ctt gaa aaa ggt gac cgg ctt tcc gct gaa att aat cgg Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg 305 310 315 320	960
cca gat tat ctt gac ttt gct gaa tcc ggt caa gtc tat ttt ggt att Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile 325 330 335	1008
att gct ctg ggc ggt ggg cag tac atc aaa gct aac tcc aaa ttc atc Ile Ala Leu Gly Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile 340 345 350	1056
ggc atc acc gaa ctg gtt cgt tct tct tct cgt acg ccg tct gat aag Gly Ile Thr Glu Leu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys 355 360 365	1104
ccg gtt gcg cac gtt gtt gcg aac ccg cag gcg gag ggg caa ttg cag Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln 370 375 380	1152
tgg ttg aat cgt cgt gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu 385 390 395 400	1200
cgt gat aac caa ttg gtt gtt ccg tct gag ggg ttg tac ttg ata tat Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr 405 410 415	1248
tct cag gtt ttg ttc aaa ggg caa ggg tgc ccg tct acg cat gtt ttg Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu 420 425 430	1296
ttg acg cac acg ata tct cgt ata gcg gtt tct tac cag acg aag gtt Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val 435 440 445	1344
aat ttg ttg tct gcg ata aaa tct ccg tgt caa cgt gaa acg ccg gaa Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu 450 455 460	1392

ggg gcg gag gcg aag ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt 1440
 Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
 465 470 475 480
 ttt cag ttg gaa aaa ggg gat cgt ttg tct gcg gag ata aac cgt ccg 1488
 Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
 485 490 495
 gac tat ttg gat ttc gcg gaa tct ggg caa gtt tac ttt ggg ata ata 1536
 Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 500 505 510
 gcg ctg taa 1545
 Ala Leu

<210> 51
 <211> 514
 <212> PRT
 <213> Artificial sequence

<220>
 <223> 3 hTNF monomers joined by tri-glycine linkers and tetanus toxoid
 P2 and P30 epitopes

<400> 51

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160

Gly Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val
 165 170 175

Ser Ala Ser His Leu Glu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp
 180 185 190

Lys Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu
 195 200 205

Gln Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu
 210 215 220

Leu Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile
 225 230 235 240

Tyr Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val
 245 250 255

Leu Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys
 260 265 270

Val Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro
 275 280 285

Glu Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly
 290 295 300

Val Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg
 305 310 315 320

Pro Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile
 325 330 335

Ile Ala Leu Gly Gly Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile
 340 345 350

Gly Ile Thr Glu Leu Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys
 355 360 365

Pro Val Ala His Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln
 370 375 380

Trp Leu Asn Arg Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu
 385 390 395 400

Arg Asp Asn Gln Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr
 405 410 415

Ser Gln Val Leu Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu
 420 425 430

Leu Thr His Thr Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val
 435 440 445

Asn Leu Leu Ser Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu
 450 455 460

Gly Ala Glu Ala Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val
 465 470 475 480

Phe Gln Leu Glu Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro
 485 490 495

Asp Tyr Leu Asp Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile
 500 505 510

Ala Leu

<210> 52

<211> 1554

<212> DNA

<213> Artificial sequence

<220>

<223> 3 hTNF monomers joined by tri-glycine linkers and with P2 and P30
 epitopes introduced

<220>

<221> CDS

<222> (1)..(1554)

<223>

<220>

<221> misc_feature
 <222> (4)..(474)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (475)..(483)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (484)..(954)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (955)..(963)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (964)..(1008)
 <223> Tetanus toxoid P2 epitope

<220>
 <221> misc_feature
 <222> (1009)..(1479)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (1480)..(1488)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (1489)..(1551)
 <223> Tetanus toxoid P30 epitope

<400>	52		
atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac			48
Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His			
1	5	10	15
gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc			96
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg			
20	25	30	
cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag			144
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln			
35	40	45	

ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 50 55 60	192
ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 65 70 75 80	240
atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 85 90 95	288
gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 100 105 110	336
aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 115 120 125	384
aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 130 135 140	432
ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 145 150 155 160	480
gga gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 165 170 175	528
gtc gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 180 185 190	576
cgg gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 195 200 205	624
ctt gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 210 215 220	672
ttt aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 225 230 235 240	720
att tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 245 250 255	768
gct att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 260 265 270	816

aaa cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 275 280 285	864
aaa ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 290 295 300	912
ttt gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 305 310 315 320	960
ggg cag tac atc aaa gct aac tcc aaa ttc atc ggc atc acc gaa ctg Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu 325 330 335	1008
gtt cgt tct tct tct cgt acg ccg tct gat aag ccg gtt gcg cac gtt Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val 340 345 350	1056
gtt gcg aac ccg cag gcg gag ggg caa ttg cag tgg ttg aat cgt cgt Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg 355 360 365	1104
gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg cgt gat aac caa ttg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu 370 375 380	1152
gtt gtt ccg tct gag ggg ttg tac ttg ata tat tct cag gtt ttg ttc Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe 385 390 395 400	1200
aaa ggg caa ggg tgc ccg tct acg cat gtt ttg ttg acg cac acg ata Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile 405 410 415	1248
tct cgt ata gcg gtt tct tac cag acg aag gtt aat ttg ttg tct gcg Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala 420 425 430	1296
ata aaa tct ccg tgt caa cgt gaa acg ccg gaa ggg gcg gag gcg aag Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys 435 440 445	1344
ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt ttt cag ttg gaa aaa Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys 450 455 460	1392
ggg gat cgt ttg tct gcg gag ata aac cgt ccg gac tat ttg gat ttc Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe 465 470 475 480	1440
gcg gaa tct ggg caa gtt tac ttt ggg ata ata gcg ctg ggt ggc gga Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly Gly 485 490 495	1488
ttc aac aac ttc acc gtt tcc ttc tgg ctg cgc gtt cca aaa gtt tcc Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser 500 505 510	1536

gct tcc cac ctg gaa taa
Ala Ser His Leu Glu
515

1554

<210> 53
<211> 517
<212> PRT
<213> Artificial sequence

<220>
<223> 3 hTNF monomers joined by tri-glycine linkers and with P2 and P30
epitopes introduced

<400> 53

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
145 150 155 160

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
165 170 175

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 180 185 190

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 195 200 205

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 210 215 220

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 225 230 235 240

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 245 250 255

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 260 265 270

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 275 280 285

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 290 295 300

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 305 310 315 320

Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 325 330 335

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 340 345 350

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 355 360 365

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 370 375 380

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 385 390 395 400

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 405 410 415

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 420 425 430

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 435 440 445

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 450 455 460

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
 465 470 475 480

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly Gly
 485 490 495

Phe Asn Asn Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser
 500 505 510

Ala Ser His Leu Glu
 515

<210> 54
 <211> 1485
 <212> DNA
 <213> Artificial sequence

<220>
 <223> 3 hTNF joined by tri-glycine linkers and PADRE added C-terminally

<220>
 <221> CDS
 <222> (1)..(1485)
 <223>

<220>
 <221> misc_feature
 <222> (4)..(474)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (484)..(954)
 <223> hTNF

<220>
 <221> misc_feature

<222> (955)..(963)
 <223> tri-glycine linker

<220>
 <221> misc_feature
 <222> (964)..(1034)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (1035)..(1043)
 <223> tri-glycine linker

<220>
 <221> misc_feature
 <222> (1044)..(1482)
 <223> PADRE

<220>
 <221> misc_feature
 <222> (475)..(483)
 <223> tri-glycine linker

<400> 54
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15
 gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30
 cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag 144
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45
 ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg 192
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60
 ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc 240
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80
 atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc 288
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95
 gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc 336
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 115 120 125	384
aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 130 135 140	432
ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 145 150 155 160	480
gga gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 165 170 175	528
gtc gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 180 185 190	576
cgg gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 195 200 205	624
ctt gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 210 215 220	672
ttt aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 225 230 235 240	720
att tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 245 250 255	768
gct att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 260 265 270	816
aaa cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 275 280 285	864
aaa ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 290 295 300	912
ttt gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 305 310 315 320	960
ggg gtt cgt tct tct tct cgt acg ccg tct gat aag ccg gtt gcg cac Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 325 330 335	1008
gtt gtt gcg aac ccg cag gcg gag ggg caa ttg cag tgg ttg aat cgt Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 340 345 350	1056

cgt gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg cgt gat aac caa 1104
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 355 360 365
 ttg gtt gtt ccg tct gag ggg ttg tac ttg ata tat tct cag gtt ttg 1152
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 370 375 380
 ttc aaa ggg caa ggg tgc ccg tct acg cat gtt ttg ttg acg cac acg 1200
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 385 390 395 400
 ata tct cgt ata gcg gtt tct tac cag acg aag gtt aat ttg ttg tct 1248
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 405 410 415
 gcg ata aaa tct ccg tgt caa cgt gaa acg ccg gaa ggg gcg gag gcg 1296
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 420 425 430
 aag ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt ttt cag ttg gaa 1344
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 435 440 445
 aaa ggg gat cgt ttg tct gcg gag ata aac cgt ccg gac tat ttg gat 1392
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 450 455 460
 ttc gcg gaa tct ggg caa gtt tac ttt ggg ata ata gcg ctg gga ggg 1440
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 465 470 475 480
 ggt gcc aag ttc gtg gcc gct tgg acc ctg aag gcc gca gct taa 1485
 Gly Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 485 490

<210> 55

<211> 494

<212> PRT

<213> Artificial sequence

<220>

<223> 3 hTNF joined by tri-glycine linkers and PADRE added C-terminally

<400> 55

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 165 170 175

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 180 185 190

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 195 200 205

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 210 215 220

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 225 230 235 240

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 245 250 255

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 260 265 270

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 275 280 285

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 290 295 300

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 305 310 315 320

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 325 330 335

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 340 345 350

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 355 360 365

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 370 375 380

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 385 390 395 400

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 405 410 415

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 420 425 430

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 435 440 445

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 450 455 460

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 465 470 475 480

Gly Ala Lys Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
 485 490

<210> 56
 <211> 1476
 <212> DNA
 <213> Artificial sequence

<220>
 <223> 3 hTNF joined by tri-glycine linkers and with PADRE in the C-term
 inus

<220>
 <221> CDS
 <222> (1)..(1476)
 <223>

<220>
 <221> misc_feature
 <222> (475)..(483)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (483)..(954)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (484)..(954)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (955)..(963)
 <223> Tri-glycine linker

<220>
 <221> misc_feature
 <222> (964)..(1434)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (1435)..(1473)
 <223> PADRE

<220>
 <221> misc_feature
 <222> (4)..(474)
 <223> hTNF

<400> 56
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

gtg gtg gcc aac ccc cag gcc gag gcc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 35 40 45	144
ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 50 55 60	192
ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 65 70 75 80	240
atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 85 90 95	288
gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 100 105 110	336
aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 115 120 125	384
aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 130 135 140	432
ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 145 150 155 160	480
gga gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 165 170 175	528
gtc gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 180 185 190	576
cgg gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 195 200 205	624
ctt gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 210 215 220	672
ttt aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 225 230 235 240	720
att tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 245 250 255	768

gct att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 260 265 270	816
aaa cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 275 280 285	864
aaa ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 290 295 300	912
ttt gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly 305 310 315 320	960
ggg gtt cgt tct tct tct cgt acg ccg tct gat aag ccg gtt gcg cac Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His 325 330 335	1008
gtt gtt gcg aac ccg cag gcg gag ggg caa ttg cag tgg ttg aat cgt Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg 340 345 350	1056
cgt gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg cgt gat aac caa Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln 355 360 365	1104
ttg gtt gtt ccg tct gag ggg ttg tac ttg ata tat tct cag gtt ttg Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu 370 375 380	1152
ttc aaa ggg caa ggg tgc ccg tct acg cat gtt ttg ttg acg cac acg Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr 385 390 395 400	1200
ata tct cgt ata gcg gtt tct tac cag acg aag gtt aat ttg ttg tct Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser 405 410 415	1248
gcg ata aaa tct ccg tgt caa cgt gaa acg ccg gaa ggg gcg gag gcg Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala 420 425 430	1296
aag ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt ttt cag ttg gaa Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu 435 440 445	1344
aaa ggg gat cgt ttg tct gcg gag ata aac cgt ccg gac tat ttg gat Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp 450 455 460	1392
ttc gcg gaa tct ggg caa gtt tac ttt ggg ata ata gcg ctg gcc aag Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Ala Lys 465 470 475 480	1440
ttc gtg gcc gct tgg acc ctg aag gcc gca gct taa Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala 485 490	1476

<210> 57
 <211> 491
 <212> PRT
 <213> Artificial sequence

<220>
 <223> 3 hTNF joined by tri-glycine linkers and with PADRE in the C-term
 inus

<400> 57

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 165 170 175

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 180 185 190

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 195 200 205

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 210 215 220

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 225 230 235 240

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 245 250 255

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 260 265 270

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 275 280 285

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 290 295 300

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 305 310 315 320

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 325 330 335

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 340 345 350

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 355 360 365

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 370 375 380

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 385 390 395 400

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 405 410 415

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
420 425 430

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
435 440 445

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
450 455 460

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Ala Lys
465 470 475 480

Phe Val Ala Ala Trp Thr Leu Lys Ala Ala Ala
485 490

<210> 58
<211> 1545
<212> DNA
<213> Artificial sequence

<220>
<223> 3 hTNF joined by glycine linkers and P2 and P30 introduced

<220>
<221> CDS
<222> (1)..(1545)
<223>

<220>
<221> misc_feature
<222> (4)..(474)
<223> hTNF

<220>
<221> misc_feature
<222> (475)..(483)
<223> tri-glycine linker

<220>
<221> misc_feature
<222> (484)..(954)
<223> hTNF

<220>
<221> misc_feature
<222> (955)..(963)
<223> tri-glycine linker

<220>
<221> misc_feature

<222> (964)..(1008)
 <223> Tetanus toxoid P2 epitope

<220>
 <221> misc_feature
 <222> (1009)..(1479)
 <223> hTNF

<220>
 <221> misc_feature
 <222> (1480)..(1542)
 <223> Tetanus toxoid P30 epitope

<400> 58
 atg gtg cgc agc agc agc cgc acc ccc agc gac aag ccc gtg gcc cac 48
 Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

gtg gtg gcc aac ccc cag gcc gag ggc caa ctg cag tgg ctg aac cgc 96
 Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

cgc gcc aac gcc ctg ctg gca aac ggc gtg gag ctg cgc gac aac cag 144
 Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

ctg gtg gtg ccc agc gag ggc ctg tac ctg atc tac agc cag gtg ctg 192
 Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

ttc aag ggc cag ggc tgc ccc agc acc cac gtg ctg ctg acc cac acc 240
 Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
 65 70 75 80

atc agc cgc atc gcc gtg agc tac cag acc aag gtg aac ctg ctg agc 288
 Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
 85 90 95

gcc atc aag agc ccc tgc cag cgc gag acc ccc gag ggc gcc gag gcc 336
 Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
 100 105 110

aag ccc tgg tac gag ccc atc tac ctc ggc ggc gtg ttc cag ctg gag 384
 Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
 115 120 125

aag ggc gac cgc ctg agc gcc gag atc aac cgc ccc gac tac ctg gac 432
 Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
 130 135 140

ttc gcc gag agc ggc cag gtg tac ttc ggc atc atc gcc ctg ggt ggc 480
 Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 145 150 155 160

gga gtc cgg tcc tcc tcc cgg aca cca tcc gac aaa cca gtc gct cat	528
Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His	
165 170 175	
gtc gtc gct aat cca caa gct gaa ggt caa ctt caa tgg ctt aat cgg	576
Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg	
180 185 190	
cgg gct aat gct ctt ctt gct aat ggt gtc gaa ctt cgg gac aat caa	624
Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln	
195 200 205	
ctt gtc gtc cca tcc gaa ggt ctt tat ctt att tat tcc caa gtc ctt	672
Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu	
210 215 220	
ttt aaa ggt caa ggt tgt cca tcc aca cat gtc ctt ctt aca cat aca	720
Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr	
225 230 235 240	
att tcc cgg att gct gtc tcc tat caa aca aaa gtc aat ctt ctt tcc	768
Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser	
245 250 255	
gct att aaa tcc cca tgt caa cgg gaa aca cca gaa ggt gct gaa gct	816
Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala	
260 265 270	
aaa cct tgg tat gaa cca att tat ctt ggt ggt gtc ttt caa ctt gaa	864
Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu	
275 280 285	
aaa ggt gac cgg ctt tcc gct gaa att aat cgg cca gat tat ctt gac	912
Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp	
290 295 300	
ttt gct gaa tcc ggt caa gtc tat ttt ggt att att gct ctg ggc ggt	960
Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly	
305 310 315 320	
ggg cag tac atc aaa gct aac tcc aaa ttc atc ggc atc acc gaa ctg	1008
Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu	
325 330 335	
gtt cgt tct tct tct cgt acg ccg tct gat aag ccg gtt gcg cac gtt	1056
Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val	
340 345 350	
gtt gcg aac ccg cag gcg gag ggg caa ttg cag tgg ttg aat cgt cgt	1104
Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg	
355 360 365	
gcg aac gcg ttg ttg gcg aat ggg gtt gaa ttg cgt gat aac caa ttg	1152
Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu	
370 375 380	
gtt gtt ccg tct gag ggg ttg tac ttg ata tat tct cag gtt ttg ttc	1200
Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe	
385 390 395 400	

aaa ggg caa ggg tgc ccg tct acg cat gtt ttg ttg acg cac acg ata 1248
 Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 405 410 415

tct cgt ata gcg gtt tct tac cag acg aag gtt aat ttg ttg tct gcg 1296
 Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 420 425 430

ata aaa tct ccg tgt caa cgt gaa acg ccg gaa ggg gcg gag gcg aag 1344
 Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 435 440 445

ccg tgg tat gaa ccg ata tac ttg ggg ggg gtt ttt cag ttg gaa aaa 1392
 Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 450 455 460

ggg gat cgt ttg tct gcg gag ata aac cgt ccg gac tat ttg gat ttc 1440
 Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
 465 470 475 480

gcg gaa tct ggg caa gtt tac ttt ggg ata ata gcg ctg ttc aac aac 1488
 Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Phe Asn Asn
 485 490 495

ttc acc gtt tcc ttc tgg ctg cgc gtt cca aaa gtt tcc gct tcc cac 1536
 Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
 500 505 510

ctg gaa taa 1545
 Leu Glu

<210> 59

<211> 514

<212> PRT

<213> Artificial sequence

<220>

<223> 3 hTNF joined by glycine linkers and P2 and P30 introduced

<400> 59

Met Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
 1 5 10 15

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
 20 25 30

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
 35 40 45

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
 50 55 60

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
65 70 75 80

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
85 90 95

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
100 105 110

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
115 120 125

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
130 135 140

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
145 150 155 160

Gly Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His
165 170 175

Val Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg
180 185 190

Arg Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln
195 200 205

Leu Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu
210 215 220

Phe Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr
225 230 235 240

Ile Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser
245 250 255

Ala Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala
260 265 270

Lys Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu
275 280 285

Lys Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp
290 295 300

Phe Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Gly Gly
 305 310 315 320

Gly Gln Tyr Ile Lys Ala Asn Ser Lys Phe Ile Gly Ile Thr Glu Leu
 325 330 335

Val Arg Ser Ser Ser Arg Thr Pro Ser Asp Lys Pro Val Ala His Val
 340 345 350

Val Ala Asn Pro Gln Ala Glu Gly Gln Leu Gln Trp Leu Asn Arg Arg
 355 360 365

Ala Asn Ala Leu Leu Ala Asn Gly Val Glu Leu Arg Asp Asn Gln Leu
 370 375 380

Val Val Pro Ser Glu Gly Leu Tyr Leu Ile Tyr Ser Gln Val Leu Phe
 385 390 395 400

Lys Gly Gln Gly Cys Pro Ser Thr His Val Leu Leu Thr His Thr Ile
 405 410 415

Ser Arg Ile Ala Val Ser Tyr Gln Thr Lys Val Asn Leu Leu Ser Ala
 420 425 430

Ile Lys Ser Pro Cys Gln Arg Glu Thr Pro Glu Gly Ala Glu Ala Lys
 435 440 445

Pro Trp Tyr Glu Pro Ile Tyr Leu Gly Gly Val Phe Gln Leu Glu Lys
 450 455 460

Gly Asp Arg Leu Ser Ala Glu Ile Asn Arg Pro Asp Tyr Leu Asp Phe
 465 470 475 480

Ala Glu Ser Gly Gln Val Tyr Phe Gly Ile Ile Ala Leu Phe Asn Asn
 485 490 495

Phe Thr Val Ser Phe Trp Leu Arg Val Pro Lys Val Ser Ala Ser His
 500 505 510

Leu Glu

<211> 2773
<212> DNA
<213> Artificial sequence

<220>
<223> p22Op2F expression vector for insect cells

<220>
<221> misc_recomb
<222> (561)..(566)
<223> HindIII site

<220>
<221> misc_recomb
<222> (573)..(578)
<223> AvaI site

<220>
<221> misc_recomb
<222> (586)..(591)
<223> EcoRI site

<220>
<221> misc_recomb
<222> (593)..(598)
<223> BamHI site

<220>
<221> misc_recomb
<222> (625)..(630)
<223> ClaI site

<220>
<221> misc_recomb
<222> (629)..(634)
<223> ClaI site

<220>
<221> misc_recomb
<222> (1156)..(1161)
<223> ApaLI site

<220>
<221> misc_recomb
<222> (2128)..(2133)
<223> PstI site

<220>
<221> misc_recomb
<222> (2204)..(2209)
<223> NcoI site

<220>
 <221> misc_recomb
 <222> (2284)..(2289)
 <223> AvaI site

<220>
 <221> misc_recomb
 <222> (2294)..(2299)
 <223> AvaI, SmaI, and XmaI site

<220>
 <221> misc_recomb
 <222> (2551)..(2556)
 <223> ApaLI site

<400> 60
 ggatcatgat gataaacaat gtatggtgct aatggttgctt caacaacaat tctgttgaac 60
 tgtgttttca tgtttgccaa caagcacctt tatactcggt ggcctcccca ccaccaactt 120
 ttttgactg caaaaaaaca cgcttttgca cgcgggccca tacatagtac aaactctacg 180
 tttcgtagac tattttacat aaatagtcta caccgttgta tacgctocaa atacactacc 240
 acacattgaa cctttttgca gtgcaaaaaa gtacgtgtcg gcagtcacgt aggccggcct 300
 tatcgggtcg cgtcctgtca cgtacgaatc acattatcgg accggacgag tgttgtctta 360
 togtgacagg acgccagctt cctgtgttgc taaccgcagc cggacgcaac tccttatcgg 420
 aacaggacgc gcctccatat cagccgcgcg ttatctcatg cgcgtgaccg gacacgaggc 480
 gccgtcccg cttatcgcg ctaaaatac agcccgaac gatctggtaa acacagttga 540
 acagcatctg ttcgaattta aagcttggtta ccctcgagct cagctgaatt ctggatcctc 600
 tagaccggtc atatgcggcc gcggatcgat cgatatctga ctaaatctta gtttgtattg 660
 tcatgtttta atacaatatg ttatgtttta atatgttttt aataaatttt ataaaataat 720
 ttcaactttt attgtaacaa cattgtccat ttacacactc ctttcaagcg cgtgggactc 780
 gatgctcact caaaggcggc aatacggtta tccacagaat caggggataa cgcaggaaag 840
 aacatgtgag caaaaggcca gcaaaaggcc aggaaccgta aaaaggccgc gttgctggcg 900
 tttttcata ggctccgccc ccctgacgag catcacaaaa atcgacgctc aagtcagagg 960
 tggcgaaacc cgacaggact ataaagatac caggcgtttc ccctggaag ctccctcgtg 1020
 cgctctcctg ttccgacct gccgcttacc ggatacctgt ccgcctttct cccttcggga 1080
 agcgtggcgc tttctcaatg ctacgctgt aggtatctca gttcgtgta ggtcgttcgc 1140

tccaagctgg gctgtgtgca cgaaccccc gttcagcccg accgctgcgc cttatccggt	1200
aactatcgtc ttgagtccaa cccggttaaga cagcacttat cgccactggc agcagccact	1260
ggtaacagga ttagcagagc gaggtatgta ggcggtgcta cagagttctt gaagtgggtg	1320
cctaactacg gctacactag aaggacagta tttggatatct gcgctctgct gaagccagtt	1380
accttcggaa aaagagttgg tagctcttga tccggcaaac aaaccaccgc tggtagcggg	1440
ggtttttttg tttgcaagca gcagattacg cgcagaaaaa aaggatctca agaagatcct	1500
ttgatctttt ctacggggc tgacgctcag tggaacgaaa actcacgtta agggattttg	1560
gtcatgatga taaacaatgt atggtgctaa tgttgcttca acaacaattc tgttgaactg	1620
tgttttcatg tttgccaaca agcaccttta tactcggtgg cctccccacc accaactttt	1680
ttgcactgca aaaaaacacg cttttgcacg cggggccata catagtacaa actctacgtt	1740
tcgtagacta ttttacataa atagtctaca ccgttgtata cgctccaaat aactaccac	1800
acattgaacc tttttgcagt gcaaaaaagt acgtgtcggc agtcacgtag gccggcotta	1860
tcgggtcgcg tcctgtcacg tacgaatcac attatcggac cggacgagtg ttgtcttacc	1920
gtgacaggac gccagcttcc tgtgttgcta accgcagccg gacgcaactc cttatcggaa	1980
caggacgcgc ctccatatca gccgcgcgtt atctcatgcg cgtgaccgga cagcaggcgc	2040
ccgtcccgtt tatcgcgcct ataaatacag cccgcaacga tctggtaaac acagttgaac	2100
agcatctgtt cgaattaatt cggatctctg cagcacgtgt tgacaattaa tcatcggcat	2160
agtatatcgg catagtataa tacgaactcac tataggaggg ccaccatggc caagttgacc	2220
agtgccgttc cgggtgtcac cgcgcgcgac gtcgccggag cggtcgagtt ctggaccgac	2280
cggctcgggt tctcccggga cttcgtggag gacgacttcg ccggtgtggt ccgggacgac	2340
gtgaccctgt tcatcagcgc ggtccaggac cagggtggtgc cggacaacac cctggcctgg	2400
gtgtgggtgc gcggcctgga cgagctgtac gccgagtggt cggaggtcgt gtccacgaac	2460
ttccgggacg cctccggggc ggccatgacc gagatcggcg agcagccgtg ggggcgggag	2520
ttcgccctgc gcgaccggc cggcaactgc gtgcacttcg tggccgagga gcaggactga	2580
ccgacgccga ccaacaccgc cggtcgcacg gcggcccacg ggtcccaggg gggtcgacct	2640
cgaaacttgt ttattgcagc ttataatggt taaaaataaa gcaatagcat cacaatttc	2700
aaaaataaag catttttttc actgcattct agttgtgggt tgtccaaact catcaatgta	2760
tcttatcatg tct	2773